

Pesticide residues in food - 2003

**Joint FAO / WHO Meeting on
Pesticide Residues**

EVALUATIONS 2003

PART II – Toxicological



IPCS

International Programme on Chemical Safety

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Pesticide residues in food—2003

WHO/PCS/04.1

Toxicological evaluations

**Sponsored jointly by FAO and WHO
With the support of the International Programme
on Chemical Safety (IPCS)**

**Joint Meeting of the
FAO Panel of Experts on Pesticide Residues
in Food and the Environment
and the
WHO Core Assessment Group**

Geneva, Switzerland, 15–24 September 2003



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* First full evaluation

** Evaluated within the periodic review programme of the Code Committee on Pesticide Residues

**2003 Joint Meeting of the FAO Panel of Experts on
Pesticide Residues in Food and the Environment
and the WHO Core Assessment Group**

Geneva, 15–24 September 2003

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Abbreviations used

ADI	acceptable daily intake
ai	active ingredient
AUC	area under the curve for concentration–time
bw	body weight
CCN	Codex classification number (for compounds or commodities)
CCPR	Codex Committee on Pesticide Residues
CYP	cytochrome P450
CXL	Codex level
DMSO	dimethyl sulfoxide
ECD	electron capture detection
F ₀	parental generation
F ₁	first filial generation
F ₂	second filial generation
FAO	Food and Agricultural Organization of the United Nations
FOB	functional observational battery
GC	gas chromatography
GCP	good clinical practice
GLC	gas–liquid chromatography
GLP	good laboratory practice
GPC	gel-permeation chromatography
GEMS/Food	Global Environment Monitoring System–Food Contamination Monitoring and Assessment Programme
GSH	glutathione
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
IEDI	international estimated daily intake
IENTI	international estimate of short-term dietary intake
ISO	International Standards Organization
JECFA	Joint Expert Committee on Food Additives
JMPR	Joint Meeting on Pesticide Residues
LC	liquid chromatography
LC ₅₀	median lethal concentration
LD ₅₀	median lethal dose
LOAEL	lowest-observed-adverse-effect level
LOAEC	lowest-observed-adverse-effect concentration
LOD	limit of detection
LOQ	limit of quantification
MDL	method detection limit
MLD	minimum level of detection
MRL	maximum residue limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level

OECD	Organization for Economic Co-operation and Development
RfD	reference dose
TMDI	theoretical maximum daily intake
UV	ultraviolet radiation
WHO	World Health Organization

Introduction

The toxicological monographs and monograph addenda contained in this volume were prepared by a WHO Core Assessment Group that met with the FAO Panel of Experts on Pesticide Residues in Food and the Environment in a Joint Meeting on Pesticide Residues (JMPR) in Geneva, Switzerland, on 15–24 September 2003.

Four of the compounds evaluated by the Core Assessment Group at the Meeting, cyprodinil, famoxadone, methoxyfenozide and pyraclostrobin, were evaluated for the first time. The other eight substances had been evaluated at previous meetings. For five of these, only information received since the previous evaluations is summarized in “monograph addenda”. Of these, dimethoate, malathion, phosmet and tebufenozide were evaluated for establishment of an acute reference dose. The appropriate earlier documents on the five compounds should be consulted in order to obtain full toxicological profiles. Toxicological monographs were prepared on carbosulfan, paraquat and terbufos, summarizing new data and, where relevant, incorporating information from previous monographs and addenda. Reports and other documents resulting from previous Joint Meetings on Pesticide Residues are listed in Annex 1.

The report of the Joint Meeting has been published by the FAO as *FAO Plant Production and Protection Paper* 176. That report contains comments on the compounds considered, acceptable daily intakes established by the WHO Core Assessment Group, and maximum residue limits established by the FAO Panel of Experts. Monographs on residues prepared by the FAO Panel of Experts are published as a companion volume, as *Evaluations 2003, Part I, Residues*, in the FAO Plant Production and Protection Paper series.

The toxicological monographs and addenda contained in this volume are based on working papers that were prepared by temporary advisers before the 2003 Joint Meeting. A special acknowledgement is made to those advisers. The monographs were edited by Dr H. Mattock, St Jean d’Ardières, France.

The preparation and editing of this volume was made possible by the technical and financial contributions of the lead institutions of the International Programme on Chemical Safety (IPCS), which supports the activities of the JMPR. The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the Central Unit of the IPCS concerning the legal status of any country, territory, city or area or of its authorities, nor concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers’ products does not imply that they are endorsed or recommended by the IPCS in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological properties or toxicity of the compounds included in this volume should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Meeting on Pesticide Residues, International Programme on Chemical Safety, World Health Organization, 20 Avenue Appia, 1211 Geneva, Switzerland.

**TOXICOLOGICAL MONOGRAPHS
AND MONOGRAPH ADDENDA**

CARBOSULFAN

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Explanation

Carbosulfan is a carbamate insecticide that acts by inhibiting the activity of acetylcholinesterase. It was evaluated by the Joint Meeting in 1984 and 1986. A toxicological monograph was prepared by the Joint Meeting in 1984 and a monograph addendum was prepared in 1986 (Annex 1, references 43, 47). In 1986, an acceptable daily intake (ADI) of 0–0.01 mg/kg bw was established on the basis of a NOAEL of 1.3 mg/kg bw per day in a 2-year study in mice, a NOAEL of 1.0 mg/kg bw per day in a 2-year study in rats, and a NOAEL of 1.3 mg/kg bw per day in a 6-month study in dogs. One of the metabolites of carbosulfan is carbofuran, which is itself used as a pesticide and which was evaluated by JMPR in 1976, 1979, 1980, 1982, 1996 and 2002. The 1996 JMPR established an ADI of 0–0.002 mg/kg bw and the 2002 JMPR established an acute reference dose (RfD) of 0.009 mg/kg bw for carbofuran.

Carbosulfan was re-evaluated by the present Meeting within the periodic review programme of the Codex Committee on Pesticide Residues. The Meeting reviewed new data on carbosulfan that had not been considered previously and relevant data from the

previous evaluation. Conclusions of studies evaluated by the JMPR in 1984 and that were not available for the present evaluation are included.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

The kinetics of [^{14}C]carbosulfan (purity, >97%) were studied in male and female Sprague-Dawley rats, in a study that complied with the United States Environmental Protection Agency (EPA) guideline 85-1. Carbosulfan was labelled either in the phenyl or in the dibutylamino moiety. In a preliminary test it was shown that excretion of radiolabel via expiration only occurred when carbosulfan was labelled in the dibutylamino group. The definitive experimental design was based on the preliminary test. Groups of five male and five female rats received unlabelled carbosulfan as a single oral dose at 4 or 30 mg/kg bw, or as repeated oral doses (4 mg/kg bw per day for 14 days), followed by [^{14}C]carbosulfan as a single oral dose at 4 mg/kg bw on day 15. Corn oil was used as vehicle. Urine, cage rinses and faeces were collected at 0–4, 4–8, 8–12, 12–24, 24–36 h intervals and every 24 h thereafter for 168 h. At 168 h after dosing, the rats were killed, blood was sampled and bone (femur), brain, fat, kidneys, liver, lungs, muscle (thighs), ovaries, testes, heart, spleen, skin (shaven, dorsal), uterus and remaining carcass were collected. For rats treated with carbosulfan labelled in the dibutylamino group, expired carbon dioxide was collected. Statements of compliance with good laboratory practice (GLP) and quality assurance (QA) were provided.

Total recovery of radioactivity was 90–98%. No marked sex differences in patterns of excretion were observed. The major route of excretion was in the urine, excretion also occurring in the faeces in expired air. Patterns of excretion were similar in rats treated with carbosulfan at a single low or high dose. In the rats treated with single low or high doses of phenyl-labelled carbosulfan, 72–83% of the radiolabel was excreted in the urine. In the groups given dibutylamine-labelled carbosulfan at a low or high dose, 65–66% and 10–17% of the radiolabel was excreted in the urine and expired air, respectively. Total excretion of radiolabel in the faeces of rats treated with single low or high doses was about 8–22%. Excretion was relatively rapid. Most (80–90%) radiolabel was excreted within 48 h in animals at the lower dose and 72 h in animals at the higher dose. Excretion of radiolabel in the urine tended to be higher (phenyl group: 79–88%, dibutylamino group 71%) in rats receiving repeated doses of carbosulfan at 4 mg/kg bw than in rats treated with a single (low) dose at 4 mg/kg bw, while excretion in faeces tended to be lower (5–15%) in rats receiving repeated doses of carbosulfan at 4 mg/kg bw, indicating that absorption was slightly increased. Furthermore, in the rats treated with repeated doses, the rate of excretion of radiolabel was increased, i.e. 80–87% of the radiolabel was excreted within 24 h in the rats treated with repeated doses versus 72–78% in the those treated with a single low dose, which indicates that induction of metabolism may have occurred. At 168 h, <0.3% of the administered dose remained in blood and tissue, and up to about 2% remained in the carcass. During the first 24 h after dosing, rats at the highest dose showed behavioural signs indicative of cholinesterase inhibition. Remarkably, a high proportion (7–11%) of administered radiolabel was found in the cage wash of animals treated with carbosulfan labelled on the phenyl group, while in animals treated with carbosulfan labelled

on the dibutylamino group only 1–3% of the radiolabel was found in the cage wash (Fang & El Naggar, 1995).

Goats

The kinetics of [^{14}C]carbosulfan was studied in lactating Nubian goats (aged 2 years; body weight, 36–43 kg), in a study that complied with EPA guideline 171-4(a)(3). Carbosulfan was labelled with ^{14}C either in the phenyl ring (purity, 99.2%) or in the dibutylamino side-chain (purity, 98.8%). Gelatin capsules containing carbosulfan at a dose of 44.7 mg/day (phenyl label) or 40.9 mg/day (dibutylamino label) (approximate dietary concentration, 25 mg/kg) were administered orally by balling gun to groups of two goats, once daily for 7 days. A single goat received only vehicle (cellulose). Urine and faeces were collected daily and milk was collected twice daily. Blood was collected on days –1, 1, 3, and 7, immediately before dosing and immediately before sacrifice. The animals were killed 22 h after the last dose and edible tissues (liver, kidney, leg and lumbar muscle, peripheral and omental fat) were collected and total radioactive residue was determined. Excretion of radiolabel in expired air was not measured. Statements of compliance with GLP and QA were provided.

The major route of elimination was in the urine. For the goats receiving carbosulfan labelled on the phenyl group, excretion of radiolabel was 81–84% in urine, 7% in faeces, 1% in cage rinse and 0.2% in milk. For the goats receiving carbosulfan labelled on the dibutylamino side-chain, the pattern of excretion was 66–70% in urine, 3–4% in faeces, 0.3–0.4% in cage rinse and 2–3% in milk. For carbosulfan labelled on the phenyl group, total radioactive residue in tissue was highest in kidney (0.15–0.21 mg/kg) and liver (0.06 mg/kg). For carbosulfan labelled on the dibutylamino side-chain, total radioactive residue was highest in omental fat (1.1–1.3 mg/kg), liver (1.0–1.3 mg/kg), kidney (0.7–0.8 mg/kg) and peripheral fat (0.7–0.8 mg/kg). In animals of both groups, concentrations of radiolabel in the blood increased over 7 days, and highest concentrations of radiolabel in blood (phenyl group, 0.02–0.04 mg/kg; dibutylamino side-chain, 0.24–0.27 mg/kg) were observed just before termination (Curry & Weintraub, 1996).

(a) Biotransformation

Rats

The metabolism of [^{14}C]carbosulfan (purity, >97%) was studied in male and female Sprague-Dawley rats in vivo, in a study that complied with EPA guideline 85-1. Carbosulfan was labelled either in the phenyl moiety or in the dibutylamino moiety. Groups of five male and five female rats received unlabelled carbosulfan as a single oral dose at 4 (low) or 30 (high) mg/kg bw, or as repeated oral doses at 4 mg/kg bw per day for 14 days, followed by [^{14}C]carbosulfan as a single oral dose at 4 mg/kg bw by gavage on day 15. Control rats received vehicle only (corn oil). Urine, cage rinses and faeces were collected at 0–4, 4–8, 8–12, 12–24, 24–36 h intervals and every 24 h thereafter for 168 h. At 168 h after dosing, the rats were killed, blood was sampled and bone (femur), brain, fat, kidneys, liver, lungs, muscle (thighs), ovaries, testes, heart, spleen, skin (shaven, dorsal), uterus and remaining carcass were collected. For rats treated with carbosulfan labelled in the dibutylamino side-chain, expired CO_2 was collected. Urine, faeces and exhaled air were analysed for radiolabel by liquid scintillation counting (LSC). Metabolites were identified by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Identity of the major metabolites was confirmed by gas chromatography-mass spectrometry (GC-MS). Statements of compliance with GLP and QA were provided.

Table 1. Quantitative excretion of carbosulfan and metabolites (% of administered radioactivity)

	Dose (mg/kg bw)											
	4 (single dose)				4 (repeated doses)				30 (single dose)			
	Urine		Faeces		Urine		Faeces		Urine		Faeces	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<i>¹⁴C-phenyl label</i>												
Carbosulfan	ND	ND	2.5	3.4	ND	ND	0.9	0.4	ND	0.3	1.7	4.4
3-OH-7-phenol	14.2	12.7	1.2	0.7	25.3	25.6	0.9	0.3	17.9	17.9	0.6	0.5
3-OH-carbofuran	11.0	10.0	6.4	3.8	17.6	20.1	4.5	1.0	16.0	11.5	1.3	1.9
3-keto-7-phenol	20.3	26.3	0.4	0.3	14.4	20.7	0.2	0.1	25.7	23.9	0.2	0.4
7-phenol	23.5	22.8	0.4	0.3	8.8	11.6	0.1	0.0	7.3	4.8	0.1	0.3
<i>¹⁴C-dibutylamino label</i>												
Carbosulfan	ND	ND	6.3	8.3	ND	ND	3.5	4.2	ND	ND	4.1	6.9
Dibutylamine	33.9	36.2	4.5	6.3	37.5	42.2	3.4	2.5	36.3	46.5	2.7	4.3
OH-dibutylamine	23.8	22.5	ND	ND	25.8	24.7	ND	ND	23.5	17.1	ND	ND

From Fang & El Naggar (1995)

ND, not detected

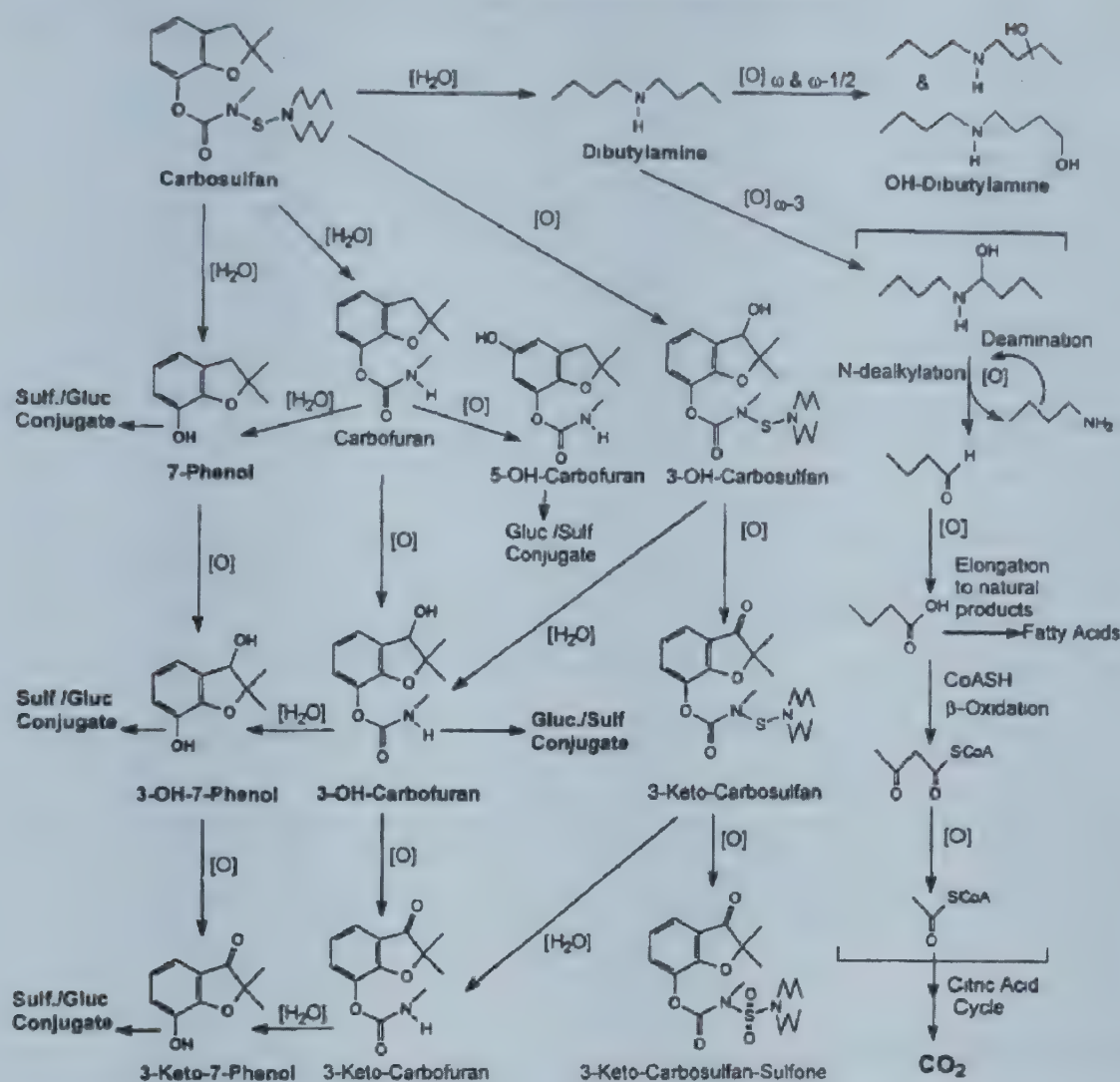
In total, 10 metabolites were identified. The distribution of radiolabel among the major metabolites is presented in Table 1. Other metabolites identified were carbofuran, 5-OH-carbofuran, 3-keto-carbosulfan, 3-OH-carbosulfan, 3-keto-carbosulfan-sulfone and 3-keto-carbofuran. Each of these metabolites or the seven unidentified metabolites was present at <5% of the total administered dose. The authors postulated that metabolites of the dibutylamino moiety may be incorporated in fatty acids. The metabolites resulting from hydrolysis and oxidation were excreted mainly as sulfate/glucuronide conjugates in urine. There was no obvious sex difference in metabolite formation. In the rats treated with single low or high doses of carbosulfan, the relative quantities of metabolites were similar. Compared with rats treated with single doses, the rats treated repeatedly with a low dose of carbosulfan had more 3-OH-7 phenol and 3-OH-carbofuran, and less 3-keto-7-phenol and 7-phenol. This may indicate that repeated dosing leads to induction of metabolizing enzyme systems. A proposed metabolic pathway is depicted in Figure 1 (Fang & El Naggar, 1995).

In a study reported in the public literature, the biotransformation of carbosulfan in the female rat stomach was assessed at short intervals after oral administration. Three rats received ¹⁴C-labelled carbosulfan, dissolved in 0.2 ml of propylene glycol, at a dose of 30 mg/kg bw. The rats were killed 15, 35 or 80 min after treatment, the stomach was removed and the stomach contents were analysed by TLC. Total recovery of radioactivity was 75.7%, 55.1% and 44.6% at 15, 35 and 80 min, respectively. Of the recovered radioactivity, 60%, 47% and 50% represented carbosulfan at 15, 35 and 80 min respectively. Major biotransformation products at 15, 35 and 80 min, respectively, were carbofuran (15%, 20% and 16%), biscarbofuran *N,N'*-disulfide (11%, 17% and 18%) and an unidentified compound (7%, 8% and 9%); the data indicate that carbosulfan is relatively stable in the stomach (Umetsu & Fukuto, 1982).

Goats

The kinetics of [¹⁴C]carbosulfan were studied in lactating Nubian goats (aged 2 years; body weight, 36–43 kg), in a study that complied with EPA guideline 171-4(a)(3). Carbosulfan was labelled with ¹⁴C, either in the phenyl ring (purity, 99.2%) or in the dibutylamino

Figure 1. Proposed metabolic pathways for carbosulfan in rats



From Fang & El Naggar (1995)

side-chain (purity, 98.8%). Gelatin capsules containing carbosulfan at a dose of 44.7 mg/day (phenyl label) or 40.9 mg/day (dibutylamino label) (approximate dietary concentration, 25 mg/kg) were administered orally by balling gun to groups of two goats, once daily for 7 days. A single goat received vehicle only (cellulose). Urine and faeces were collected daily and milk was collected twice daily. Blood was collected on days -1, 1, 3, and 7, immediately before dosing and immediately before sacrifice. The animals were killed 22 h after the last dose and edible tissues (liver, kidney, leg and lumbar muscle, peripheral and omental fat) were collected. Samples of milk and tissues were extracted and metabolites were identified by HPLC and TLC; additional information was sometimes obtained by size-exclusion chromatography and GC-MS. Statements of compliance with GLP and QA were provided.

The major metabolites of phenyl- or dibutylamino-labelled carbosulfan are presented in Table 2. The metabolites containing the phenyl label were found in conjugated, protein-bound or non-conjugated form. Greater quantities were found of metabolites containing the dibutylamino label than of metabolites containing the phenyl label. This is attributed to the fact that a large part of the dibutylamino residue is broken into smaller fragments and incorporated into natural components, such as fatty acids, triglycerides, carbohydrates and protein (Curry & Weintraub, 1996).

Table 2. Quantitative distribution of metabolites of carbosulfan in goats (% of total radioactive residue in the sample)

Metabolite	Sample				
	Milk	Liver	Kidney	Fat	Muscle
<i>¹⁴C-phenyl label</i>					
3-OH-carbofuran	34.2	9.5	21.5	ND	ND
3-OH-7-phenol	21.1	15.6	13.3	ND	ND
3-keto-7-phenol	29.9	3.0	9.3	ND	ND
7-phenol	9.2	4.6	8.9	ND	ND
Minor metabolites	1.2	4.4	7.6	ND	ND
Characterized organosolubles	2.3	17.3	17.4	ND	ND
Protein-associated metabolites	NA	22.6	2.5	ND	ND
Polar aqueous metabolites	0.7	10.4	18.4	ND	ND
Non-extractable residues	1.4	12.7	2.1	ND	ND
<i>¹⁴C-dibutylamino label</i>					
Aminobutanols	29.7	8.1	11.9	0.8	ND
Dibutylamine and related metabolites	6.7	13.4	10.5	0.6	9.6
Natural constituents	30.2	29.1	13.8	87.3	32.0
Non-conjugated amines	11.8	6.3	24.3	ND	5.9
Conjugated or bound amines	10.5	18.0	12.3	ND	14.7
Lipophilic metabolites	0.6	1.3	4.5	0.5	1.2
Polar aqueous metabolites	7.6	16.6	18.5	0.2	26.5
Non-extractable residues	2.9	7.2	4.2	10.5	10.0

From Curry & Weintraub (1996)

NA, not applicable; ND, not detected

2. Toxicological studies

2.1 Acute toxicity

(a) Oral, dermal and inhalation administration

For the present evaluation, no studies of acute toxicity with technical-grade carbo-sulfan were available. All available studies of oral, inhalatory and dermal toxicity were performed with formulations of carbosulfan that contained 25–50% active ingredient (a.i.). Data on the composition of the formulations were not provided in the studies. The acute toxicity of formulations containing carbosulfan is summarized in Table 3. The LD₅₀s for the formulations and the recalculated values for the active ingredient are presented, assuming that the active ingredient is responsible for the toxic effect. In most of the studies with carbo-sulfan administered orally, the LD₅₀ tended to be lower for females than for males. In general, the clinical signs observed resemble those of cholinesterase inhibition. Studies of acute toxicity with technical-grade carbosulfan were evaluated by the JMPR in 1984. The oral LD₅₀s in rats ranged from 90 to 250 mg/kg bw. The LD₅₀ for carbosulfan was >2000 mg/kg bw in rabbits treated dermally, and the LC₅₀ was 0.61 mg/l for carbosulfan in rats treated by inhalation.

The Meeting noted that in the studies of dermal toxicity the formulation was moistened with water or saline. In view of the high lipophilicity of carbosulfan (log p_{ow} = 5.4), it is not certain that good contact with the skin was maintained with the vehicles used.

(b) Ocular irritation

For the present evaluation, no studies of ocular irritation with pure or technical-grade carbosulfan were available. Five studies of ocular irritation with formulations of carbo-sul-

Table 3. Acute toxicity of formulations containing carbosulfan

Species	Strain	Sex	Formulation code ^a	Purity (%)	Route and vehicle	LD50 (mg/kg bw) or LC50 (mg/l)		Reference
						Formulation	Active ingredient	
Mouse	SW	Male	25 ST	28.9	Oral, in water	1077	311	Freeman (1985b) ^b
		Female				869	251	
Rat	SD	Male	4 EC	49.7	Oral, undiluted	69	34	Seaman (1981) ^d
		Female				≤55	≤27	
Rat	COBS(SD)	Male & Female	40 DB	40	Oral, in corn oil	>50	>20	Sabol (1981a) ^d
Rat	SD	Male	40 DB	40	Oral, in corn oil	348	134	Freeman (1984a) ^b
		Female				159	64	
Rat	SD	Male	25 ST	28.9	Oral, in water	278	80	Freeman (1985a) ^b
		Female				107	31	
Rat	Wistar	Male	35 STD	35	Oral, in water	113	40	Daamen (1991a) ^b
		Female				147	51	
Rat	SD	Male	25 STW	25	Oral, in water	258	65	Freeman (1989a) ^b
		Female				120	30	
Rat	Him:OFA	Male & Female	25 CS	25	Oral, in water	238	60	Klein (1993a) ^b
Rat	Wistar	Male & Female	400 SC	40.3	Oral, in water	42	17	Mello dos Santos (1998a) ^c
Rat	Wistar	Male & Female	35 STD	35	Dermal, in water	>2000	>700	Daamen (1991b) ^b
Rat	Him:OFA	Male & Female	25 CS	25	Dermal, undiluted	>2000	>700	Klein (1993b) ^b
Rat	Wistar	Male	400 SC	40.3	Dermal, in water	563	227	Mello dos Santos (1998b) ^c
		Female				688	269	
Rabbit	NZW	Male & Female	40 BD	40	Dermal, in saline	>200	>80	Sabol (1981b) ^d
Rabbit	NZW	Male & Female	25 ST	28.9	Dermal, in saline	>2000	>578	Freeman (1985c) ^b
Rabbit	NZW	Male & Female	25 STW	25	Dermal, in saline	>2000	>500	Freeman (1989b) ^b
Rat	CrI:CD	Male & Female	40 DB	40	Inhalation (1 h) ^h	<5.0	<2	Morgan (1981) ^d
Rat	CrI:CD	Male & Female	4 EC	47.2	Inhalation (1 h) ^d	<2.2	<1	Horath (1982) ^b
Rat	SD	Male & Female	25 STW	25	Inhalation (4 h) ^a	>0.11	>0.028	Blagden (1997) ^b
Rat	SD	Male	25 ST	28.9	Inhalation (4 h) ^f	0.26	0.075	Dudek (1985)
		Female				0.10	0.029	
Rat	SD	Male & Female	25 STD	35	Inhalation (1 h) ⁱ	>0.43	>0.15	Signorin (1993) ^b
Rat	Wistar	Male	400 SC	40.3	Inhalation (4 h) ^g	1.37	0.55	Mello dos Santos (1998c) ^c
		Female				1.72	0.69	

NSW, New Zealand white; SD, Sprague-Dawley

^a All formulations are named "Marshal", followed by the codes indicated in the table^b Statements of compliance with GLP and QA were provided^c A statement of compliance with GLP was provided^d A statement of compliance with QA was provided^e Nose only; mass median aerodynamic diameter (MMAD), 7.2 µm; inhalable fraction, 16.6%^f Whole body; MMAD not presented^g Nose only; actual concentration and MMAD aerosol not measured^h Whole body; actual concentration and MMAD not assessedⁱ Whole body; gravimetric concentration, 0.43 mg/l; MMAD, 8.74 µm

tion were performed. The proportion of active ingredient in these formulations ranged from 25 to 40.3%.

In the studies of ocular irritation in rabbits, performed in compliance with guideline 81-4 and OECD 405, the irritating properties of Marshal 25 STW (25% a.i.), Marshal 25 ST (28.9% a.i.), Marshal 25 CS (25% a.i.), Marshal 400 SC (40.3% a.i.) and Marshal 35 STD (35% a.i.) were assessed. The conclusions of these studies were that Marshal 25 STW is minimally irritating to the eye, Marshal 35 STD is mildly irritating to the eye and that the other formulations do not induce ocular irritation (Freeman, 1985d, 1989d; Pels Rijcken, 1991a; Klein, 1993d; Mello dos Santos, 1998e). Statements of compliance with GLP and QA were provided.

(c) *Dermal irritation*

For the present evaluation by the Meeting, no studies of dermal irritation with pure or technical-grade carbosulfan were available. Five studies on dermal irritation with formulations of carbosulfan were performed. The level of active ingredient in these formulations ranged from 25 to 40.3%.

In the studies of dermal irritation in rabbits, performed in compliance with guideline 81-5 or OECD 404, the irritating properties of Marshal 25 STW (25% a.i.), Marshal 25 ST (28.9% a.i.), Marshal 25 CS (25% a.i.), Marshal 400 SC (40.3% a.i.) and Marshal 35 STD (35% a.i.) were assessed. The conclusions of these studies were that Marshal 25 ST is minimally irritating to the skin, Marshal 400 SC is slightly irritating to the skin, and that the other formulations do not induce dermal irritation (Freeman, 1985e, 1989e; Pels Rijcken 1991b; Klein, 1993c; Mello dos Santos, 1998f). Statements of compliance with GLP and QA were provided.

(d) *Dermal sensitization*

For the present evaluation by the Meeting, no studies of sensitization with pure or technical-grade carbosulfan were available. Four studies on the skin-sensitizing properties of formulations of carbosulfan were performed. The level of active ingredient in these formulations ranged from 25 to 40.3%.

In three standard Buehler assays for skin sensitization, performed according to guideline 81-6, the sensitizing properties of 0.3 g of Marshal 25 STW (25% a.i.), 0.4 g of Marshal 25 ST (28.9% a.i.), and 0.4 g of Marshal 40 DB (40% a.i.) were assessed. The formulations were moistened with saline and the above-mentioned quantities were used in induction as well as in the challenge phase. None of the formulations induced dermal sensitization. Statements of compliance with GLP and QA were provided (Freeman, 1984b, 1985f, 1989c).

A study of dermal sensitization in guinea-pigs (three males, two females; strain unknown) that received intradermal applications of Marshal 400 SC (40.3% a.i.) was considered to be inadequate by the Meeting, since this test was not performed according to the currently required guidelines, the number of animals was insufficient to perform a sound statistical evaluation, and the methods and results sections were poorly described (Mello dos Santos, 1998d).

2.2 *Short-term studies of toxicity*

(a) *Dermal administration*

Rabbits

In a study that complied with EPA guideline 82-2, groups of six male and six female New Zealand white rabbits received carbosulfan (purity, 91.6%) at a dose of 0, 50, 200 or 800 mg/kgbw per day for 21 consecutive days in daily applications, under an occlusive wrapping, to the clipped skin of the back (doses were selected on the basis of a range-finding study). The test material, being a liquid, was applied undiluted to the skin, covering about 5%, 10–15% or 75% of the test site in the groups receiving the lowest, intermediate and highest dose, respectively. Animals in the control group were treated in the same manner, but received no test material. Each day, the test material was removed 6 h after application by wiping the treated skin with a gauze pad moistened with methanol and subsequently rinsing it with tepid tap water. The animals were observed daily for mortality, clinical signs or signs of dermal toxicity. Body weight and food consumption were measured weekly. After 21 days, the rabbits were killed and necropsies were performed. One female in the group receiving the highest dose died on day 3. Data from this animal were not included in the analysis and the animal was replaced. Clinical chemistry was performed before the start of treatment and at termination. Plasma and erythrocyte cholinesterase activities were determined before initiation of the study and on days 10 and 21, during the 6 h period of exposure. Cholinesterase activity in the brain was determined at termination, within 2 h after the end of the exposure. Statements of compliance with GLP and QA were provided.

One male and two female rabbits in the groups receiving the highest dose died during the study. The death of one animal was attributed to bronchopneumonia. The cause of death of the other two animals could not be established, but was not considered to be treatment-related. Some rabbits in the group receiving the lowest dose had soft stools. In the animals in the groups receiving the intermediate and highest doses, diarrhoea was frequently observed. In the latter group, one male and several females displayed decreased limb tone, and nasal discharge was observed in two females. All treatment groups showed slight erythema (incidence was dose-dependent) and oedema. No signs of dermal irritation were observed in the control animals. Body weight and food consumption were not affected. Apart from inhibition of cholinesterase activity, no treatment-related haematological and biochemical changes were observed. Organ weights were not affected. Except for dermal effects at the site of application, no microscopic changes were observed. The effect of carbosulfan on cholinesterase activity is summarized in Table 4.

At the highest dose (800 mg/kgbw), erythrocyte cholinesterase activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain cholinesterase activity. No consistent sex differences in cholinesterase inhibition were found. On the basis of the statistically significant reduction in erythrocyte cholinesterase activity (22%) in females at the intermediate dose, and the statistically not significant decrease in brain cholinesterase activity (30% and 22% in males and females respectively) at the intermediate dose, the study author considered the NOAEL to be 50 mg/kgbw per day (Goldenthal, 1990a).

The Meeting noted that it was not specified at which time-point during the 6 h period of exposure the blood samples for determination of erythrocyte and plasma cholinesterase

Table 4. Effects of carbosulfan on cholinesterase activity^a in rabbits after dermal application

Substrate		Dose (mg/kg bw)					
		50		200		800	
		Males	Females	Males	Females	Males	Females
Erythrocytes	Day 10	86	91	94	94	94	75
	Terminal	84	88	78*	84	84	72*
Plasma	Day 10	63*	75	38*	25*	25*	38*
	Terminal	60*	80	40*	20*	20*	30*
Brain	Terminal	90	78	78	40*	40*	44*

From Goldenthal (1990a)

^a As cholinesterase activities varied considerably over time, cholinesterase activity of animals in the treatment groups is expressed as per cent of the value for concurrent controls rather than as per cent of the pretest value

* Significantly different from control

activity were drawn, and no data on the peak plasma concentrations of carbosulfan are available. Furthermore, it was reported that brain cholinesterase activity was assessed “within 2 h after the end of exposure”. The effects of carbamates being known to be transient, blood and brain concentrations of carbosulfan at this time may be below peak concentrations. Therefore, measurements of plasma, erythrocyte and brain cholinesterase activity in this study may underestimate the level of inhibition induced by carbosulfan. The variability in the assay and the relatively small group size may explain why decreases of 20–30% in cholinesterase activity were often not statistically significant. In a second study by the same author (Goldenthal, 1990b), the lowest-observed-adverse-effect level (LOAEL) was 50 mg/kg bw per day in male rabbits given carbosulfan by dermal application (see below). In view of the potential underestimation of the inhibition of cholinesterase activity and the small number of animals per group in the present study, and taking into account data from the second study by Goldenthal, the Meeting considered that the statistically non-significant reduction (22%) in cholinesterase activity in the brain of females at 50 mg/kg bw (the lowest dose) to be an adverse effect. Therefore, the LOAEL for carbosulfan in this study was 50 mg/kg bw per day.

In a study performed in compliance with EPA guideline 82-2, groups of six male New Zealand white rabbits were given carbosulfan (purity, 91.6%) at a dose of 0, 5, 50 or 100 mg/kg bw per day for 21 consecutive days by daily dermal application, under an occlusive wrapping, to the clipped skin of the back (doses were selected on the basis of a range-finding study). The test material, being a liquid, was applied undiluted to the skin, covering <5%, 5% and 10% of the test site in animals in the groups receiving the lowest, intermediate and highest doses, respectively. Animals in the control group were treated in the same manner, but received no test material. Each day, the test material was removed 6 h after application, by wiping the treated skin with a gauze pad moistened with methanol and subsequently rinsing it with tepid tap water. The animals were observed daily for mortality, clinical signs or signs of dermal toxicity. Body weight and food consumption were measured weekly. After 21 days, the rabbits were killed and necropsies were performed. Clinical chemistry was performed before the start of treatment and at termination. Plasma and erythrocyte cholinesterase activity was determined before the initiation of the study and on days 10 and 21, during the 6 h period of exposure. Cholinesterase activity in one-half brain sections was determined at termination, within 2 h after the end of the exposure. Statements

Table 5. Effects of carbosulfan on cholinesterase activity^a in male rabbits after dermal application

Substrate	Time-point	Dose (mg/kg bw)		
		5	50	200
Erythrocytes	Day 10	81	86	74
	Terminal	93	100	90
Plasma	Day 10	89*	67*	67*
	Terminal	75*	63*	63*
Brain	Terminal	92	77*	69*

From Goldenthal (1990b)

^a As cholinesterase activities varied considerably over time, cholinesterase activity of animals in the treatment groups is expressed as per cent of the value for concurrent controls rather than as per cent of the pretest value

* Significantly different from control

of compliance with GLP and QA were provided. No clinical signs of toxicity were observed. Dose-related slight (all treatment groups) to moderate (highest dose) erythema and oedema were observed. Body weight and food consumption were not affected. No treatment-related haematological and biochemical changes were observed. Organ weight was not affected. Except for dermal effects at the site of application, no microscopic changes in the organs were observed. The effects of carbosulfan on cholinesterase activity are summarized in Table 5.

Erythrocyte cholinesterase activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain cholinesterase activity. On the basis of the statistically significant reduction in brain cholinesterase activity in animals in the group receiving 50 mg/kg bw per day, the NOAEL was 5 mg/kg bw per day (Goldenthal, 1990b).

The Meeting noted that it was not specified at which time-point during the 6 h period of exposure the blood samples for determination of erythrocyte and plasma cholinesterase activity were drawn, and no data on the peak plasma concentrations of carbosulfan are available. Furthermore, it is reported that brain cholinesterase activity was assessed “within 2 h after the end of exposure”. The effects of carbamates being known to be transient, blood and brain concentrations of carbosulfan may be below peak concentrations at this time. Therefore, measurements of plasma, erythrocyte and brain cholinesterase activity in this study may underestimate the level of inhibition induced by carbosulfan.

(b) Inhalation

Rats

Groups of five male and five female Sprague-Dawley rats received whole-body exposure to air containing liquid droplets of carbosulfan technical (purity, 89.6%) stabilized in epoxidized soybean oil, 6 h per day, for 5 days. Mean actual concentrations to which the animals were exposed were 5.4, 15.6, and 47 µg/l. A control group was exposed to room air. An additional group of five rats of each sex were killed before treatment to establish cholinesterase activity before exposure to the test material. During exposure, the groups of animals were observed regularly for clinical signs. Detailed observations of individual animals were carried out before and after exposure. Body weights were measured before exposure on day 1 and after exposure on day 5. After the exposure on day 5, the animals

were killed and necropsies were performed, and cholinesterase activity was determined in erythrocytes, plasma and brain. Histopathology was not performed. Statements of compliance with GLP and QA were provided.

The mass median aerodynamic diameter (MMAD) ranged from 1.9 to 2.6 μm . Effects were observed in all treatment groups, these effects increasing in incidence and severity with increasing dose. Females tended to be more sensitive to exposure to carbosulfan than males. During exposure, fasciculation was observed in all treatment groups. Animals in the groups receiving the intermediate and highest doses also displayed irregular breathing and decreased activity. Additionally, salivation, lacrimation, tremors and anogenital staining were observed in the group receiving the highest dose. After exposure, anogenital staining, decreased activity, lacrimation and irregular breathing were observed to occur in a dose-dependent manner in all groups. On day 5, body weights in all treatment groups tended to be less than those of controls; this effect reached statistical significance in males at the highest dose. Except for discoloration of the anogenital and nasal area, no treatment-related macroscopic changes in the organs were observed. The effects of carbosulfan on cholinesterase activity are summarized in Table 6.

Erythrocyte cholinesterase activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain cholinesterase activity. No consistent sex differences in cholinesterase inhibition were found. Owing to the reduction in brain cholinesterase activity and the clinical signs observed during and after exposure in the groups receiving the lowest dose, a no-observed-adverse-effect concentration (NOAEC) could not be identified. The lowest-observed-adverse-effect concentration (LOAEC) was 5.4 mg/l (Whitman, 1990a).

The Meeting noted that the animals were killed within 3 h 20 min after the end of the final exposure. The effects of carbamates being known to be transient, blood and brain plasma concentrations of carbosulfan at this time may be below peak concentrations. Therefore, measurements of plasma, erythrocyte and brain cholinesterase activities in this study may underestimate the level of inhibition induced by carbosulfan.

Groups of 10 male and 10 female Sprague-Dawley rats received whole-body exposure to air containing liquid droplets of carbosulfan technical (purity, 89.6%) stabilized in epoxidized soybean oil, 6 h per day, 5 days per week for 3 weeks. Mean actual concentra-

Table 6. Effects of carbosulfan on cholinesterase activity^a in rats exposed by inhalation for 5 days

Substrate	Dose ($\mu\text{g/l}$)					
	5.4		15.6		47	
	Males	Females	Males	Females	Males	Females
Erythrocyte	95	95	92*	90*	94	87*
Plasma	61*	53	60*	42*	49*	37*
Brain	59*	52*	54*	52*	38*	40*

From Whitman (1990a)

^a% of value for controls

* Significantly different from control

tions to which the animals were exposed were 0.15, 0.65 and 5.34 µg/l. An additional group of 10 rats of each sex was killed before treatment, to establish cholinesterase activity before exposure. During exposure, the groups of animals were observed hourly for clinical signs. Detailed observations of individual animals were carried out before and after exposure. Body weights were measured before study initiation, weekly at the start of the exposure, and before and after exposure on the last day. Ophthalmological examinations were performed before study initiation and during the last week of exposure. Within 3 h 20 min after the last exposure, the animals were killed and necropsies were performed. Blood and brain tissue were collected for haematology and clinical chemistry, including determination of cholinesterase activity in erythrocytes, plasma and brain. Organs were dissected and weighed, and microscopical examination was performed on a full range of organs and tissues. Statements of compliance with GLP and QA were provided. The MMAD ranged from 1.9 to 2.0 µm. No clinical signs were observed in animals at the lowest and intermediate doses. At the highest dose, fasciculations, decreased activity, irregular and laboured breathing, salivation, lacrimation, and dried nasal discharge, anogenital staining, soft stool were observed during or after exposure. No treatment-related effects on ophthalmology and body weight were observed. Occasional findings on macroscopical and histopathological examinations, on organ weights and on blood urea nitrogen values were considered to be incidental and not related to treatment. The effects of carbosulfan on cholinesterase activity are summarized in Table 7.

Erythrocyte cholinesterase activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain cholinesterase activity. No consistent sex differences in cholinesterase inhibition were found. Although statistically significant, only small decreases in brain cholinesterase activity were observed at the intermediate dose (7% in males, 11% in females). Moreover, no clinical signs of toxicity were observed in this group. On the basis of the occurrence of clinical signs and inhibition of brain cholinesterase activity in animals at the highest dose, the NOAEC was 0.65 µg/l (Whitman, 1990b).

The Meeting noted that the animals were killed within 5 h 52 min after the end of the final exposure. The effects of carbamates being known to be transient, plasma concentrations of carbosulfan at this time may be below peak concentrations. Therefore, measurements of plasma, erythrocyte and brain cholinesterase activity in this study may underestimate the level of inhibition induced by carbosulfan.

Table 7. Effects of carbosulfan on cholinesterase activity^a in rats exposed by inhalation for 5 days

Substrate	Dose (µg/l)					
	0.15		0.65		5.34	
	Males	Females	Males	Females	Males	Females
Erythrocyte	101	95	100	96	92	91
Plasma	100	81	97	102	64*	63*
Brain	98	98	93*	89*	57*	64*

From Whitman (1990b)

^a% of value for controls

* Significantly different from control

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 100 male and 100 female Charles River CD-1 mice were given diets containing carbosulfan (purity, 94.5–95.6%, with 0.6–2.4% carbofuran) at a concentration of 0, 10, 20, 500 or 2500 mg/kg (equal to 0, 1.3, 2.5, 62 and 320 mg/kg bw per day for males, and 0, 1.5, 3.1, 72 and 337 mg/kg bw per day for females) for 24 months. All animals were observed daily for signs of toxicity, moribundity and mortality. Body weight and food consumption values were recorded weekly for the first 14 weeks and every two weeks thereafter. Water consumption was determined monthly. Haematological and biochemical measurements and urine analyses, including cholinesterase activity, were performed for 10 unfasted mice of each sex per group at the 6, 12, 18 and 24 month sacrifices. Ophthalmoscopic evaluations were performed in all survivors at 24 months. Selected organs were weighed, gross necropsies conducted and a complete list of tissues and organs examined microscopically. A statement of compliance with QA was provided.

There were no measurable treatment-related effects on mortality or survival. Mean body weights were reduced throughout the study for males at 500 and 2500 mg/kg and for females at 2500 mg/kg. For females, body-weight changes at other dietary concentrations were sporadic and not related to treatment. Males at 20 mg/kg, however, had decreased body-weight gains from week 42 to terminal sacrifice at 104 weeks. Food consumption was depressed for males and females at 2500 mg/kg, but only sporadic decreases were noted at other dietary concentrations.

There were no measurable differences with regard to general appearance and behaviour, except for increased eye irregularities at 2500 mg/kg. These included corneal opacity, eccentric pupil, and white, cloudy eyes. There were no treatment-related effects or haematological changes, except for a tendency towards slightly increased segmented neutrophils and decreased lymphocyte counts in males at 2500 mg/kg. There were no demonstrated effects on glucose, blood urea nitrogen, alanine aminotransferase or alkaline phosphatase in either sex at any dose. The effects of carbosulfan on cholinesterase activity are summarized in Table 8. Since the observed inhibition of cholinesterase activity in the treated groups was not dependent on the time of sampling (i.e. 6, 12, 18 and 24 months), the mean cholinesterase activity (expressed as a percentage of the control value) and range of the means for the four time-points are shown in Table 8.

Cholinesterase activity for plasma, erythrocytes and brain tissue was significantly depressed in both males and females at 500 and 2500 mg/kg, at all time-points.

Ophthalmoscopic examinations indicated an increase in punctuate opacities of the iris at 500 and 2500 mg/kg. Females in the group receiving the highest dose were observed to have focal retinal degeneration. An apparent difference of opinion was expressed by two ophthalmologists, regarding the incidence of cataracts in male mice. An independent ophthalmological examination was subsequently performed, the results of which were considered by the JMPR in 1986. It was concluded that there was no evidence for cataractogenesis in the study of carcinogenicity in mice. Therefore, the concern expressed by the Meeting in 1984 with regard to the cataractogenic potential of carbosulfan was alleviated. Special evaluation and concern for the iris, because of compound-related effects in the rat (DeProspero et al., 1982b) did not indicate similar effects in mice.

Table 8. Effects of carbosulfan on cholinesterase activity^a in mice after administration in the diet

Substrate	Dietary concentration (mg/kg)			
	10	20	500	2500
<i>Males</i>				
Erythrocytes	98 (91–104)	92 (89–96)	64 (56–73)*	52 (40–63)*
Plasma	93 (86–100)	95 (86–100)	52 (36–73)*	38 (36–41)*
Brain	100 (89–107)	105 (93–114)	51 (39–57)*	35 (32–43)*
<i>Females</i>				
Erythrocyte	96 (90–100)	93 (88–98)	66 (64–70)*	56 (48–60)*
Plasma	99 (85–109)	96 (79–105)	77 (72–81)*	49 (41–53)*
Brain	101 (98–102)	97 (88–106)	50 (35–79)*	40 (29–61)*

From DeProspero et al. (1982a)

^a Overall mean % activity, relative to controls (range of means)

* Significantly different from control

Absolute organ weight changes were variably affected in both males and females, except for decreased spleen weight in females at 500 and 2500 mg/kg. Relative spleen weights were also significantly decreased in females at 500 and 2500 mg/kg. Relative brain weights were significantly increased throughout the study for both sexes at 2500 mg/kg. This is considered a reflection of the significant effects on body weight at the higher doses.

The results of gross and histopathological examinations were essentially unremarkable. The most common findings reported were malignant lymphoma and bronchioalveolar adenoma, which were equally distributed among all groups, except for females at the lowest dose; the latter had a significant increase in the number of metastatic malignant lymphomas of mediastinal and mesenteric lymph nodes, as well as thymus and spleen. Generally, the incidence of malignant lymphomas was highest in females in the control group and in the group receiving the lowest dose. The results of the histopathological examination indicated that the type and incidence of non-neoplastic and neoplastic lesions were normal findings in the mouse and were unrelated to treatment. Carbosulfan was not carcinogenic in mice at dietary concentrations up to and including 2500 mg/kg, equal to 320 and 337 mg/kg bw per day for males and females respectively.

On the basis of the decrease in body weight, the NOAEL was 10 mg/kg, equal to 1.3 mg/kg bw per day (DeProspero et al., 1982a).

Rats

Groups of 90 male and 90 female Charles River CD rats were given diets containing carbosulfan (purity, 94.5–95.6%) at a concentration of 0, 10, 20, 500 or 2500 mg/kg (equal to 0, 0.5, 1.0, 27 and 153 mg/kg bw for males, and 0, 0.6, 1.2, 35 and 213 mg/kg bw for females) for 104 weeks. Carbofuran was present in the technical material at a concentration of 0.6–2.4%. Growth was observed by body-weight changes and data on food consumption that were recorded weekly for the first 14 weeks and every two weeks thereafter. Daily observations were made with respect to behavioural changes and mortality. At periodic intervals throughout the study, haematological, biochemical and cholinesterase analyses were performed on unfasted animals. Urine analysis was conducted on fasted animals.

Eyes were examined at 12, 18 and 24 months. At 6, 12 and 18 months of the study, 10 males and 10 females per group were sacrificed and necropsies were performed. At the termination of the study, all surviving animals were sacrificed and gross pathological and microscopic examinations of tissues and organs were made, and selected organs were weighed. A statement of compliance with QA was provided.

Tremors, laboured breathing and eye-related changes were more frequently observed in animals receiving the diets containing carbosulfan at a concentration of 500 and 2500 mg/kg. Mean body weight and food consumption values of animals at 500 and 2500 mg/kg were significantly lower than control values throughout the study, except for female food consumption, which was comparable to controls. Survival was not apparently affected by treatment. Measurement of haematology parameters at 6, 12, 18 and 24 months demonstrated a compound-related effect at 18 months in males and females at 2500 mg/kg, including a significantly increased leukocyte count (primarily segmented neutrophils), and platelet count, slightly increased reticulocyte count and significantly decreased lymphocyte count. After 24 months, haemoglobin, erythrocyte cell volume, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were all lower, although not significantly, in males at the highest dose than in controls. After 18 months, urine analysis showed that males and females at the highest dose had higher concentrations of ketones than did controls. At terminal sacrifice, there was an increase in mononuclear leukocyte infiltrates in the kidney in animals receiving the intermediate and highest doses, and an increase in pigmentation (haemosiderin) in the mediastinal lymph nodes of treated females. All these data are indicative of leukocytosis and toxic neutrophilia. During early phases of neutrophilia, there is a tendency toward acidosis, which is demonstrated by the presence of ketone bodies in the urine. The elevated platelet count is also a reflection of hyperactivity of the bone marrow. However, histopathology of bone marrow, spleen and liver was otherwise unremarkable. Biochemical analyses were generally comparable with those for controls, except for males and females at the highest dose: decreased albumin, total protein and globulin were reported in these animals. Plasma, erythrocyte and brain cholinesterase activity was significantly decreased in males and females at a concentration of 500 or 2500 mg/kg. Significantly increased relative brain, heart, liver and kidney weights in males and females at the intermediate and highest doses were attributed to lower body weights. Absolute spleen, adrenal and thyroid weights were uniformly lower for groups receiving carbosulfan at 500 and 2500 mg/kg, but were not different from those of controls on an organ-to-body weight basis. Carbosulfan produced compound-related effects on the eye, which were described pathologically as focal iris atrophy, iris coloboma and absence of iris tissues in males and females at 500 and 2500 mg/kg, as well as degenerative retinopathy in females at 2500 mg/kg. The atrophy of the iris was attributed, in part, to an extensive anticholinesterase effect. There were no treatment-related effects on the eye at 10 or 20 mg/kg. Gross and histopathologic examinations of all tissues, except the eye, revealed no compound-related effects on the incidence or type of neoplastic or non-neoplastic changes. Carbosulfan was not carcinogenic to rats at dietary concentrations of up to and including 2500 mg/kg (equal to 153 and 213 mg/kgbw per day for males and females, respectively, the highest dose tested). The NOAEL was 20 mg/kg, equal to 1 mg/kgbw per day, on the basis of pathology of the eye, clinical signs and cholinesterase inhibition (DeProspero et al., 1982b).

2.4 Genotoxicity

A range of tests for genotoxicity in vitro and in vivo was performed with carbosulfan or formulations of carbosulfan. A number of these studies were reported in the public

literature. Some were poorly described and did not comply with current guidelines, GLP or QA. Positive effects were reported in a number of studies from public literature. The results of tests for genotoxicity are summarized in Table 9.

A test for sister chromatid exchange in human lymphocytes (Rencüzoğullari & Topaktas, 1996) was deemed to be inadequate, since positive controls were lacking and the test was only performed in the absence of metabolic activation. A study in which the genotoxicity of mixtures of carbosulfan and other substances were tested was not evaluated (Rencüzoğullari & Topaktas, 2000). One study that reported negative effects with carbosulfan in an Ames test and positive effects in a test with *Saccharomyces cerevisiae* D61.M was not evaluated, because only the abstract was available (Wiedenmann et al., 1990).

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of 15 male and 30 female Charles River CD rats were given diets containing carbosulfan at a concentration of 0, 10, 20 and 250 mg/kg (equivalent to 0, 0.67, 1.3 and 16.7 mg/kg bw per day) for three generations. Two successive litters were reared from each female. General condition and behaviour were observed routinely and individual body weights were recorded throughout the study. The number of pups in each litter was examined and pups were culled to 10 per litter at age 4 days. Individual pup weights were measured on days 0, 4, 7, 14 and 21. Ten male and 10 female F₁b, F₂b, and F₃b weanlings were randomly selected for gross necropsy and tissue collection. The F₁a and F₂a litters were discarded at weaning, and the F₁b and F₂b litters were used to produce succeeding generations. Weanlings not selected for continuation in the study (F₁a, F₂a, F₁b, F₂b, F₃a and F₃b) were subjected to gross external examination and sacrificed and discarded. A statement of compliance with QA was provided.

Body weights of F₀ males and females at 20 and 250 mg/kg showed initial decreases. These decreases were associated with reduced food consumption, and both recovered to normal levels between week 4 and sacrifice. Body weights of parental males (F₁ and F₂) receiving the diet containing carbosulfan at 250 mg/kg were consistently lower than those of animals in the corresponding control group. A similar effect was observed in females at 250 mg/kg at sometimes during gestation and lactation, but not during the growth phases. Mating index, gestation index and number of viable fetuses were essentially normal throughout the study, except for F₂b dams at 250 mg/kg, for which values for these parameters were decreased. Litter size, pup weights and/or pup weight gains for all litter sets in the group receiving carbosulfan at 250 mg/kg were significantly lower at most age intervals between birth and weaning than for the concurrent control group. Neonatal survival at 250 mg/kg was also significantly lower for the first four litters (F₁a, F₁b, F₂a and F₂b). There were no treatment-related gross or histological changes observed among the F₀, F₁b and F₂b adults or the F₁b, F₂b and F₃b weanlings. Carbosulfan did not have an adverse effect on reproductive performance. Pup weight, litter size, and pup survival was decreased at 250 mg/kg. In parental animals, the NOAEL was 20 mg/kg (equivalent to 1.3 mg/kg bw per day), on the basis of the decreases in body weight. The NOAEL for fetotoxicity was 20 mg/kg (equivalent to 1.3 mg/kg bw per day) on the basis of reductions in litter size, pup weight and pup weight gain. The NOAEL for reproductive toxicity was 250 mg/kg (equivalent to 16.7 mg/kg bw per day, the highest dose tested) (Kehoe & MacKenzie, 1982).

Table 9. Results of studies of genotoxicity with carbosulfan

End-point	Test object	Concentration	Purity (%)	Results	Reference
<i>In vitro</i>					
Reverse mutation	<i>Bacillus subtilis</i> H-17 (rec ⁺), M-45 (rec ⁻)	0.0005–2 mg/plate	NR	Negative	Jagannath (1979) ^c
Reverse mutation	<i>S. typhimurium</i> . TA98, TA100, TA1535, TA1537, TA1538	0.1–10 µl/plate	93	Negative	Haworth et al. (1980) ^a
Reverse mutation	<i>E. coli</i> . WP2, CM611 <i>S. typhimurium</i> . TA1978, TA1538	0.025, 0.05, 0.1 & 0.2 ml/plate	93	Negative	Haworth et al. (1981) ^{a,d}
Reverse mutation	<i>S. typhimurium</i> . TA97a, TA98, TA100, TA1535	0.001–5 mg/plate	40.3	Negative	Gava (1988) ^{a,e}
Gene mutation	Mouse lymphoma L5178Y <i>Tk</i> ^{h/-}	0.0024–0.032 µl/ml, –S9 0.0056–0.075 µl/ml, +S9	93	Negative	Kirby et al. (1981) ^a
Chromosomal aberration	Human lymphocytes	1 × 10 ⁻⁶ to 5 × 10 ⁻⁵ dilution (v/v)	NR	Positive	Topaktas & Rencüzoğullari (1993) ^f
Sister chromaid exchange	Human lymphocytes	25, 50, 100 µmol/l	NR	Positive	Rencüzoğullari & Topaktas (1998) ^g
Gene conversion, reverse mutation, aneuploidy	<i>S. cerevisiae</i> D7, D61.M	4.9–980 µmol/l	93	Negative	Stehrer-Schmid & Wolf (1995) ^h
<i>In vivo</i>					
Micronucleus formation	Mouse bone-marrow cells	43.5 & 87 mg/kg bw 20.3, 40.6, & 60.9 mg/kg bw (twice, 24 h interval)	93	Negative	Kirkhart et al. (1979) ^{b,i}
Micronucleus formation	Mouse bone-marrow cells	20.3, 40.6 & 60.9 mg/kg bw (twice, 24 h interval)	40.3	Negative	Franco Perina (1998) ^{a,j}
Micronucleus formation	Mouse bone-marrow cells	29.6, 59.2 mg/kg bw	93	Positive	Stehrer-Schmid & Wolf (1995) ^k
Chromosomal aberration	Rat bone-marrow cells	0, 5, 12, 30 mg/kg bw for 5 consecutive days	93	Negative	Putnam & Schechtman (1981) ^{b,l}
Chromosomal aberrations	Rat bone-marrow cells	12.5, 25, 50 mg/kg bw	NR	Positive	Topaktas & Rencüzoğullari (1996) ^m
Dominant lethal mutation	Mice	0, 7, 20, 60 mg/kg bw for 5 consecutive days	93	Negative	Preache et al. (1981) ^{a,n}

Positive and negative (solvent) controls were included in all studies

NR, not reported; S9, 9000 × g supernatant of induced rat liver

^a Statements of compliance with GLP and QA were provided

^b A statement of compliance with QA was provided

^c Test substance, FMC 35001. S9 fraction of Aroclor 1254-induced rat liver

^d Preferential kill of repair-deficient *E. coli* CM611 without liver microsomes, at 0.025–0.2 ml/plate

^e Test substance was Marshal 400SC. S9 fraction of Aroclor 1254-induced rat liver

^f Study from the public literature. Both carbosulfan (purity not reported) and a formulation of carbosulfan, identified as Marshal (not further specified) were tested. Chromosomal aberrations were reported at all concentrations of carbosulfan and at the two highest concentrations of Marshal

^g Study from the public literature. Carbosulfan (purity not reported) induced sister chromatid exchange after 48 h treatment but not after 24 h

^h Study from the public literature

ⁱ Test compound was administered orally. A dose of 174 mg/kg bw caused excess mortality and was excluded

^j Marshal 400SC was administered intraperitoneally, in two doses of 75, 50 and 25% of the LD₅₀, separated by a 24 h interval. No deaths were reported. The ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE:NCE) was not affected (at the highest dose, carbosulfan induced a 14% decrease in the PCE:NCE ratio in males)

^k Study from the public literature. Carbosulfan was administered intraperitoneally. Treatment with carbosulfan resulted in a modest, time-dependent increase in micronucleus formation

^l Test compound was administered orally in single doses

^m Study from the public literature. A formulation of carbosulfan, identified as Marshal (not further specified) was administered intraperitoneally. Chromosomal aberrations were reported at all concentrations and time-points

ⁿ The test compound was administered orally. Parameters evaluated were fertility index, total number of implantations, number of live implantations, number of early and late implantation deaths, body weights and clinical signs

(b) *Developmental toxicity*

Rats

Groups of 25 female Charles River CD rats were given carbosulfan at a dose of 0, 2, 10 and 20 mg/kg bw per day by oral gavage on days 6–19 of gestation. Necropsies were performed on surviving females on day 20 and on fetuses delivered by hysterotomy. The number and position of viable/non-viable fetuses, early/late resorptions, mean number of corpora lutea and total number of implantations were recorded. External, internal and skeletal examinations of fetuses were performed. One-third of the fetuses were evaluated for soft-tissue anomalies and the remaining two-thirds for skeletal effects. A statement of compliance with QA was provided.

There was no dose-related increase in mortality in any treated group. Body tremors were reported at 20 mg/kg bw per day after administration of each dose. Clear oral discharge was observed in dams at 10 and 20 mg/kg bw per day. Mean maternal body-weight gains were slightly reduced at 10 mg/kg bw per day and significantly reduced at 20 mg/kg during gestation. There were no differences in number of pregnancies, early/late resorptions, viable fetuses, postimplantation loss or sex distribution. The number of corpora lutea per dam was increased in all treated groups, while the mean fetal body weight was significantly reduced at 10 and 20 mg/kg bw per day. There was also an increase in the number of litters with developmental variations at 20 mg/kg bw per day; these variations included reduced ossification of the skull, unossified hyoid body, unossified sternbrae 5 and 6, and undeveloped renal papilla and/or distended ureters. There were no reported effects on the number or per cent of fetuses or litters with external, internal or skeletal malformations or anomalies at any dose. On the basis of the clinical signs and reduction in body weight, the NOAEL for maternal toxicity was 2 mg/kg bw per day. On the basis of the reduction in fetal body weight, the NOAEL for fetotoxicity was 2 mg/kg bw per day. The NOAEL for developmental toxicity was 20 mg/kg bw per day (the highest dose tested) (Janes et al., 1980a).

Rabbits

Groups of 16 New Zealand albino rabbits were given carbosulfan at a dose of 0, 2, 5, and 10 mg/kg bw per day by gavage on days 6–28 of gestation. Pups were delivered by caesarean section on day 29, and the number, location, and distribution of viable/non-viable fetuses, corpora lutea, early/late resorptions and total implantations were recorded. All fetuses were examined grossly, sectioned for visceral anomalies and stained for skeletal anomalies. A statement of compliance with QA was provided.

No treatment-related effects on appearance and behaviour were observed in the dams. There were three deaths of animals treated at 10 mg/kg, one death in the control group and one death in the group receiving the lowest dose. A cause of death could not be established for two dams in the group receiving the highest dose and in the dam in the group receiving the lowest dose. The deaths of one of the dams at the highest dose and the dam at the lowest dose were attributed to enteritis. The numbers of dams with viable fetuses were 12, 10, 15 and 11 at 0 (control), 2, 5 and 10 mg/kg, respectively. There were no compound-related effects on the number of viable fetuses, corpora lutea, fetal sex distribution or total implantations per dam. There were slight, non-significant increases in postimplantation losses (6, 9, 11 and 13 at 0, 2, 5 and 10 mg/kg bw per day, respectively) and early resorption rate (3, 8, 9 and 11 at 0, 2, 5 and 10 mg/kg bw per day, respectively). Also, the mean fetal body weight was slightly decreased (7%) at the highest dose. There was a single

occurrence of scoliosis in each of the three treated groups, but none was reported in the controls. There were no compound-related effects on skeletal variations, such as delayed ossification. Major vessel variations, identified primarily as left carotid arising from the innominate, were observed in 16.7, 100, 46.7 and 72.7% of the litters at 0, 2, 5, and 10 mg/kg bw per day, respectively (mean of data for historical controls in the test laboratory, 51%). The proportions of fetuses presenting this defect were 4.9, 44, 12.8 and 20.8%, respectively (mean of data for historical controls in the test laboratory, 14%). The increase in major vessel variations was not dose-dependent. Furthermore, the Meeting noted that data for historical controls (MARTA, 2003) show that these major vessel variations are very common and occur at highly variable incidences in rabbits of this strain. Therefore the Meeting considered these effects to be incidental. The NOAEL for maternal and fetotoxicity was 10 mg/kg bw per day (the highest dose tested). Carbosulfan was not teratogenic in rabbits. The NOAEL for developmental toxicity was 10 mg/kg bw per day (the highest dose tested) (Janes et al., 1980b).

2.6 *Special studies*

(a) *Neurotoxicity*

(i) *Acute neurotoxicity*

The neurotoxic effects of acute oral exposure to carbosulfan (purity, 88%) were assessed in groups of 27 male and 27 female Crl:CD BR rats treated by gavage in a study that complied with EPA guideline 81-8-SS. The animals received vehicle (corn oil), or carbosulfan at a dose of 0.5, 5 or 30 mg/kg bw. All animals were checked daily for viability and clinical signs. Body weights were measured on days -6, 0, 7, 14 and 15. The doses and time-points were selected on the basis of a preliminary study. Seven animals of each sex per dose were allocated to undergo neuropathological examination on day 15; this consisted of assessment of brain weight and dimensions, evaluation for gross changes, and histopathological examination of a range of central and peripheral nervous system tissues (including eyes, ganglions and nerve fibres). In groups of five animals of each sex per dose, brain, erythrocyte and plasma cholinesterase activities were measured before treatment, and 4 h after dosing on days 0, 7 and 15. Additionally, at termination the brains of animals in these four groups were weighed, and six brain regions (see Table 10) were dissected. The animals that were allocated to undergo neuropathological examination and the animals for which cholinesterase activity was measured on day 15 were also tested in a functional observational battery (FOB) and for locomotor activity before treatment, 4 h after dosing, and on days 7 and 14. Statements of compliance with GLP and QA were provided.

No mortality was observed. During the first week after treatment, body-weight gain was lower in males at the highest dose. Also at the highest dose, some animals had staining of the ventral abdomen and urogenital area on days 1 and 2. Home cage observations revealed one male at the highest dose with tremors on day 0. At 4 h after dosing, body temperatures were decreased in males and females at the highest dose, a few animals with slight tremors and impaired gait were observed during open-field testing, and sensory tests revealed a slow tail-pinch response in males. Furthermore, motor activity was decreased in animals at the highest dose. Effects on brain weight, gross changes and histopathology were considered not to be treatment-related. Data on cholinesterase activity at 4 h after dosing in the groups treated with carbosulfan are summarized in Table 10.

Table 10. Effects of carbosulfan on cholinesterase activity^a in rats, 4 h after dosing by oral gavage

Substrate	Dose (mg/kg bw)					
	0.5		5		30	
	Males	Females	Males	Females	Males	Females
<i>Blood</i>						
Plasma	96	135	76*	116	61*	88
Erythrocytes	101	89	62*	64*	54*	55*
<i>Brain</i>						
Hippocampus	86	108	73	62*	54*	62*
Olfactory region	93	88	66	66*	52*	58*
Midbrain	97	92	64*	61*	53*	53*
Brain stem	84	94	56*	59*	48*	52*
Cerebellum	95	94	74*	67*	61*	68*
Cortex	88*	94	62*	66*	52*	51*

From Knapp (1996)

^a% of value for controls

*Significantly different from control

In females, plasma cholinesterase activity appeared to be less sensitive than erythrocyte and brain cholinesterase activity to inhibition by carbosulfan. Brain and erythrocyte cholinesterase activity was reduced in males and females at the intermediate and highest doses, 4 h after administration of carbosulfan. No consistent differences in cholinesterase activity were observed before treatment or on days 7 and 14. Occasional significant differences at these time-points were considered not to be treatment-related.

On the basis of the effects on cholinesterase activity in brain and erythrocytes at the intermediate dose, the NOAEL was 0.5 mg/kg bw (Knapp, 1996).

(ii) Neurotoxicity after repeated doses

Groups of 10 male and 10 female Sprague-Dawley CD rats were given diets containing carbosulfan (purity, 88.0%) at a concentration of 0, 20, 1000 or 2000 mg/kg (equal to 0, 1.2, 65 and 131 mg/kg bw per day in males, and 0, 1.4, 79 and 152 mg/kg bw per day in females) for 13 weeks, in a study that complied with EPA guidelines 81, 82 and 83. Clinical signs were recorded daily. Body weights and food consumption were measured weekly. The animals were subjected to FOB and motor activity tests before treatment and 4, 8 and 13 weeks after the start of treatment. At termination, the animals were killed and necropsies were performed. The nervous systems of five animals of each sex in the control group and in the group receiving the highest dose were examined for neuropathological lesions. Statements of compliance with GLP and QA were provided.

At the intermediate and highest doses (dietary concentrations, 1000 or 2000 mg/kg respectively), body weight and body-weight gains were decreased in males and females. At these doses, food consumption in males was decreased throughout the study and food consumption in females was decreased during weeks 2 and 3. Chromodacryorrhoea and decreased faeces were also observed in males at these doses. Additionally, tremors, exophthalmus, chromorhinorrhoea and dehydration were observed in males at the highest dose. In females at the intermediate and highest doses, exophthalmus, tremors, abdominogenital

staining, reddish brown staining of the cage-pan liner, chromorhinorrhoea, chromodacryorrhoea, decreased faeces, dehydration, diarrhoea and unthriftiness were observed. In the FOB, males at the highest dose (2000 mg/kg) showed decrease in foot splay, hindlimb grip strength and urine pools. In females at the highest dose, localized spasms/twitching or tremors, exophthalmos, walking on toes, and slightly impaired gait were observed. A reduction in motor activity was observed in females at the two higher doses at weeks 4 and 13 respectively. No treatment-related effects on necropsy and neuropathological examination were found. Measurements of cholinesterase activity in brain, erythrocyte or plasma were not included in this study. On the basis of the clinical signs, observations in the FOB, and effects on body weight, body-weight gain and food consumption in animals at the two higher doses, the NOAEL was 20 mg/kg, equal to 1.2 mg/kg bw per day (Freeman, 1995).

(b) Effects of carbosulfan on semen characteristics and serum testosterone

A study reported in the public literature described the effects of carbosulfan on semen characteristics and serum testosterone concentrations in male rabbits. The study report was confounded by lack of information on the purity of the compound, the doses used, and data on body weight and nutritional state of the animals. Therefore, the Meeting concluded that the significance of the reported effects could not be established (El-Zarkouny et al., 1999).

Comments

The absorption of radiolabelled carbosulfan administered orally is rapid and almost complete in male and female rats. Elimination is also relatively rapid; most (80–90%) of the absorbed radioactivity is excreted in the urine within 48–72 h, depending on the dose administered. After repeated dosing of rats with carbosulfan, the rate of excretion appeared to be increased (80–87% within 24 h), which may indicate that induction of metabolism has occurred.

Carbosulfan is metabolized by hydrolysis to the 7-phenol or to carbofuran and dibutylamine, and is subsequently further metabolized via hydrolysis, oxidation and conjugation to a variety of metabolites. Metabolites of the dibutylamino moiety may enter the carbon pool and be incorporated into natural constituents of the body. No marked sex-specific differences were observed in rats with regard to the excretion pattern, tissue distribution and metabolite profile of carbosulfan.

Carbosulfan (technical material) is highly toxic when administered orally, with LD₅₀s ranging from 90 to 250 mg/kg bw in rats. The LD₅₀ for carbosulfan was >2000 mg/kg bw in rabbits treated dermally and the LC₅₀ was 0.6 l mg/l in rats treated by inhalation.

Carbosulfan is minimally irritating to the eye, slightly irritating to the skin and is a dermal sensitizer.

In general, in short-term and long-term studies of toxicity, the most sensitive effect of the oral administration of carbosulfan was the inhibition of cholinesterase activity, accompanied at the same or higher doses by clinical signs indicative of cholinesterase inhibition (e.g. salivation, lacrimation, ataxia, tremors, anogenital staining, diarrhoea). In a study of acute oral neurotoxicity in rats, the NOAEL was 0.5 mg/kg bw on the basis of effects on brain cholinesterase activity as measured 4 h after dosing. In a 90-day study in rats, the NOAEL was 20 mg/kg, equivalent to 1 mg/kg bw per day on the basis of inhibition of brain

and erythrocyte cholinesterase activity. In a second 90-day study of rats fed with carbosulfan, the NOAEL was 20 mg/kg, equal to 1.2 mg/kg bw per day, on the basis of clinical signs, observations in FOB and effects on body weight, body-weight gain and food consumption at a dose of 62 mg/kg bw per day. In this study, cholinesterase activity was not determined.

In a 6-month study in dogs, the NOAEL was 50 mg/kg, equivalent to 1.3 mg/kg bw per day, on the basis of effects on blood chemistry parameters and occasional reductions in food consumption and body-weight gain.

In long-term studies in mice and rats, carbosulfan was not carcinogenic at dietary concentrations of up to and including the highest dose tested, 2500 mg/kg, equal to 320 and 153 mg/kg bw per day for mouse and rat, respectively. In the study in mice, the NOAEL was 20 mg/kg, equal to 2.5 mg/kg bw per day, on the basis of reductions in body weight, inhibition of brain and erythrocyte cholinesterase activity and reductions in absolute and relative spleen weight. In the study in rats, the NOAEL was 20 mg/kg, equal to 1 mg/kg bw per day, on the basis of inhibition of brain and erythrocyte cholinesterase activity and pathological changes in the eye, i.e. focal iris atrophy, iris coloboma and absence of iris tissue. The mechanism by which these pathological changes in the eye were induced is not clear.

The genotoxic potential of carbosulfan was investigated in a wide range of tests. Primarily negative results were obtained in a number of tests in vitro and in vivo. Positive effects were observed in a few tests, however these tests were confounded by the use of very high doses in vivo, the occurrence of marked cytotoxicity in vitro and the lack of information on the purity of the test compound. The Meeting concluded that carbosulfan is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in rats and mice the Meeting concluded that carbosulfan is unlikely to pose a carcinogenic risk to humans.

In a three-generation study of reproductive toxicity, carbosulfan was administered at a dose of 10, 20 or 250 mg/kg of diet. No effects on mating index, gestation index and number of viable fetuses were observed. At 250 mg/kg, pup weight, litter size and pup survival were decreased, as were the body weights of parental males and females at this dose. In parental animals, the NOAEL was 20 mg/kg, equivalent to 1.3 mg/kg bw per day, on the basis of the decreases in body weight. The NOAEL for pup toxicity was 20 mg/kg on the basis of the reductions in litter size, pup body weight and pup body-weight gain. The NOAEL for reproductive toxicity was 250 mg/kg, equivalent to 17 mg/kg bw per day, the highest dose tested.

In studies of developmental toxicity in rats and rabbits, carbosulfan was not teratogenic. The NOAEL for maternal toxicity was 2 mg/kg bw per day in the study in rats, on the basis of clinical signs and reduction in body weight. The NOAEL for toxicity in offspring was 2 mg/kg bw per day, on the basis of the reduction in fetal body weight. In the study in rabbits, the NOAEL for maternal and offspring toxicity was 10 mg/kg bw per day, the highest dose tested.

When tested in hens, carbosulfan did not induce delayed polyneuropathy after a single exposure, according to a study evaluated by the Meeting in 1984.

No new data were available for humans.

The Meeting concluded that the present database is sufficient to characterize the potential hazard of carbosulfan to fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.01 mg/kg bw per day based on a NOAEL of 1 mg/kg bw per day, on the basis of pathological changes in the eye, inhibition of brain and erythrocyte cholinesterase activity and body-weight reduction in the 2-year study in rats, with a safety factor of 100. This safety factor was used because the pathological changes in the eye could not definitely be attributed to inhibition of cholinesterase.

After considering the data available to the present Meeting, as well as the previous evaluations, the Meeting established an acute RfD of 0.02 mg/kg bw. This was based on the NOAEL of 0.5 mg/kg bw per day for inhibition of brain cholinesterase activity in a study of acute neurotoxicity in rats, and a safety factor of 25, as the relevant toxic effects of carbosulfan are dependent on the C_{\max} (Annex 1, reference 95).

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	2-year study of toxicity and carcinogenicity ^a	Toxicity	20 mg/kg, equal to 2.5 mg/kg bw per day	500 mg/kg, equal to 62 mg/kg bw per day
		Carcinogenicity	2500 mg/kg, equal to 320 mg/kg bw per day ^c	
Rat	Three-generation study of reproductive toxicity ^a	Parental and offspring toxicity	20 mg/kg, equivalent to 1.3 mg/kg bw per day	250 mg/kg, equivalent to 17 mg/kg bw per day
		Reproductive toxicity	250 mg/kg, equivalent to 17 mg/kg bw per day ^c	—
	Study of developmental toxicity ^b	Maternal toxicity	2 mg/kg bw per day	10 mg/kg bw per day
		Embryo- and fetotoxicity	2 mg/kg bw per day	10 mg/kg bw per day
	Study of acute neurotoxicity ^b	Neurotoxicity	0.5 mg/kg bw	5 mg/kg bw
	90-day study of neurotoxicity ^a	Neurotoxicity	20 mg/kg, equivalent to 1 mg/kg bw per day	500 mg/kg, equivalent to 25 mg/kg bw per day
		Toxicity	20 mg/kg, equal to 1 mg/kg bw per day	500 mg/kg, equal to 27 mg/kg bw per day
	2-year study of toxicity and carcinogenicity ^a	Carcinogenicity	2500 mg/kg, equal to 153 mg/kg bw per day ^c	—
Rabbit	Study of developmental toxicity ^b	Maternal toxicity	10 mg/kg bw per day ^c	—
		Embryo- and fetotoxicity	10 mg/kg bw per day ^c	—
Dog	6-month study of toxicity ^a	Toxicity	50 mg/kg, equivalent to 1.3 mg/kg bw per day	500 mg/kg, equivalent to 13 mg/kg bw per day

^a Diet

^b Gavage

^c Highest dose tested

Estimate of acceptable daily intake for humans

0–0.01 mg/kg bw

Estimate of acute reference dose

0.02 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

Further observations in humans

Summary of critical end-points for carbosulfan*Absorption, distribution, excretion and metabolism in animals*

Rate and extent of absorption	Rapid and extensive
Dermal absorption	No data (rabbit: reduction in brain cholinesterase activity at 50 mg/kg bw per day)
Distribution	Extensive; highest concentrations in liver, kidney, omental fat, peripheral fat
Potential for accumulation	Low
Rate and extent of excretion	Relatively rapid (80–90% within 48–72 h in rats, mainly in urine)
Metabolism in animals	Major metabolites: 3-OH-7-phenol, carbofuran, 3-OH-carbofuran, 3-keto-7-phenol, 7-phenol, dibutylamine (rat)
Toxicologically significant compounds	Carbosulfan, carbofuran

Acute toxicity

Rat, LD ₅₀ , oral	90–250 mg/kg bw
Rabbit, LD ₅₀ , dermal	>2000 mg/kg bw
Rat, LC ₅₀ , inhalation	0.61 mg/l
Rabbit, dermal irritation	A mild irritant
Rabbit, ocular irritation	A mild irritant
Dermal sensitization	Sensitizing (Buehler)

Short-term studies of toxicity

Target/critical effect	Inhibition of cholinesterase activity in brain and erythrocytes
Lowest relevant oral NOAEL	1 mg/kg bw per day (rats)
Lowest relevant dermal NOAEL	5 mg/kg bw per day (rabbits)
Lowest relevant inhalatory NOAEC	0.00065 mg/l (rats)

Genotoxicity

Negative in most tests; unlikely to be genotoxic

Long-term studies of toxicity and carcinogenicity

Target/critical effect	Inhibition of cholinesterase activity in brain and erythrocytes, pathological changes in the eye
Lowest relevant NOAEL	1 mg/kg bw per day (rats)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans

Reproductive toxicity

Reproduction target/critical effect	Reduction of pup weight, litter size and pup survival (in the presence of parental toxicity)
Lowest relevant reproductive NOAEL	1.3 mg/kg bw per day (rats)
Developmental target	Reduction in pup weight (in the presence of maternal toxicity)
	Not teratogenic
Lowest relevant developmental NOAEL	2 mg/kg bw per day (rats)

Neurotoxicity/delayed neurotoxicity

Neurotoxicity	Inhibition of cholinesterase activity in brain and erythrocytes, and clinical and behavioural effects associated with cholinesterase inhibition
Lowest relevant oral NOAEL	0.5 mg/kg bw (rats)
Delayed neurotoxicity	Negative

Medical data

None

Summary	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw	Rat, long-term toxicity	100
Acute RfD	0.02 mg/kg bw	Rat, acute neurotoxicity	25

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CYPRODINIL

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Explanation

Cyprodinil is the ISO approved name for (4-cyclopropyl-6-methyl-pyrimidin-2-yl)-phenyl-amine, a systemic fungicide that acts by inhibiting the biosynthesis of methionine. Cyprodinil has not been evaluated previously by the JMPR.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution, and excretion

(a) Oral administration

Rats

Groups of Tif RAIf (SPF) rats were given a single oral dose of radiolabelled cyprodinil (purity, $\geq 98\%$) at 0.5 or 100 mg/kg bw. Cyprodinil was labelled either in the phenyl ring or on the pyrimidyl group; the positions of the radiolabel, ^{14}C , are shown in Figure 1. One group (C1) was given unlabelled cyprodinil (purity, $\geq 99\%$) at a dose of 0.5 mg/kg bw for 2 weeks before being treated with the radiolabelled compound. Groups of five male and female rats were given a suspension of radiolabelled cyprodinil in ethanol/polyethyleneglycol 200/water (1:2:1, v/v) by gavage. The design of the study is described in Table 1. The absorption, distribution and elimination of cyprodinil administered intravenously were not evaluated, owing to the limited solubility of the test substance in aqueous media.

The absorption of cyprodinil was estimated from the total amount of radiolabel excreted in the bile and urine in animals with bile cannulae and receiving a higher dose (group G1). In the 48 h after dosing, 39% (range, 27.5–43.4%) of the administered dose was eliminated in the bile and 35.4% (range, 27.8–44.2%) was eliminated in the urine. Therefore, the total estimated absorption of cyprodinil was 75.5% (range, 71–85%) of the administered dose within 48 h.

The blood kinetics of radiolabelled cyprodinil, measured in animals in group E1, indicated an initial rapid absorption, with a peak concentration of cyprodinil equivalents ($C_{\text{max}} = 0.083 \pm 0.036 \text{ mg/kg}$) being reached 15 min after administration. The concentration of cyprodinil equivalents in the blood declined rapidly to one-half of the maximum by 1.25 h after administration. A second prolonged phase of absorption showed a peak ($0.029 \pm 0.019 \text{ mg/kg}$) at about 8 h after administration; this was probably caused by reabsorption of radiolabel that was excreted in the bile. At 48 h, the mean concentration of cyprodinil equivalents in the blood was $0.0017 \pm 0.0001 \text{ mg/kg}$.

Figure 1. Position of radiolabel on cyprodinil used in a study of absorption, distribution and excretion in rats

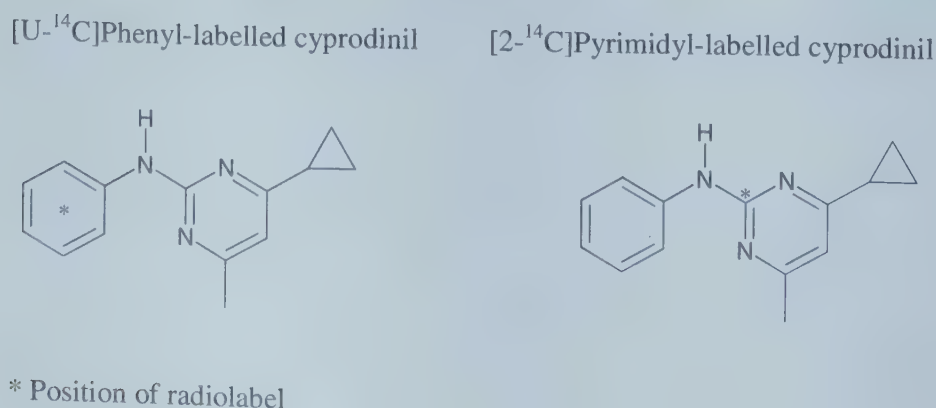


Table 1. Design of a study of absorption, distribution and excretion of radiolabelled cyprodinil in rats treated by gavage

Group	No. of animals and sex	Dose		Times at which samples were collected
		(mg/kg bw)	(kBq)	
B1	5 males and 5 females	0.5 ^a	185	Urine: 0–8, 8–24, and every 24 h thereafter until 168 h Faeces: 0–24, and every 24 h thereafter until 168 h Tissues: 168 h
C1 ^c	5 males	0.45 ^a	185	Urine: 0–8, 8–24, and every 24 h thereafter until 168 h
	5 females	0.52 ^a	185	Faeces: 0–24, and every 24 h thereafter until 168 h Tissues: 168 h
D1	5 males	94.3 ^a	7247	Urine: 0–8, 8–24, and every 24 h thereafter until 168 h
	5 females	93.7 ^a	7247	Faeces: 0–24, and every 24 h thereafter until 168 h Expired air: 0–24, 24–48 h Tissues: 168 h
D2	5 males	102.2 ^b	7313	Urine: 0–8, 8–24 and every 24 h thereafter until 168 h
	5 females	100.4 ^b	7313	Faeces: 0–24, and every 24 h thereafter until 168 h Expired air: 0–24, 24–48 h Tissues: 168 h
E1	3 males	0.51 ^a	183	Blood: 0.25, 0.5, 1, 2, 4, 8, 12, 24, 32, 48 h
F1	4 × 3 males	0.54 ^a	188	Tissues: 0.25, 1.25, 11, 17 h
	4 × 1 males	—	—	
G1	5 males	106.5 ^a	9406	Urine: 0–24, 24–48 h Faeces: 0–24, 24–48 h Bile: 0–0.5, 0.5–1, 1–2, 2–4, 4–8, 8–18, 18–24, 24–42, 42–48 h

From Thanei (1992)

^a[U-¹⁴C]Phenyl label

^b[2-¹⁴C]Pyrimidyl label

^cPretreated with unlabelled cyprodinil, 14 × 0.5 mg/kg bw per day

Seven days (168 h) after the administration of single oral doses at 0.5 mg/kg bw (group B1), the mean tissue concentration of cyprodinil equivalents was below the limit of determination or at the limit of detection of radioactivity, except in liver, kidney, spleen (B1 females only), whole blood (B1 males or females) and remaining carcass (B1 females only). Concentrations of cyprodinil equivalents in selected tissues of the B1 males and females were as follows: liver, 0.0046–0.0048 mg/kg; kidneys, 0.0031–0.0061 mg/kg; spleen, 0–0.0015 mg/kg; blood, 0.0011–0.0012 mg/kg and remaining carcass, 0–0.0017 mg/kg. Residues in the tissues of animals that had received repeated doses at 0.45 and 0.52 mg/kg bw for males and females, respectively (group C1; pretreated with unlabelled cyprodinil) were of the same order of magnitude as those in animals that had received a single dose (group B1). Tissue residues were low in the groups that were given a high single dose (groups D1 and D2); the concentration of cyprodinil equivalents did not exceed 0.2 mg/kg, except in kidneys, liver, lungs, spleen (females only), thyroid, whole blood, and remaining carcass (<1.5 mg/kg). The distribution and concentrations of the two different radiolabelled compounds were similar (groups D1 and D2). Tissue residues in females were 0.5–2.7-fold greater than in males, and total tissue residues for animals in both treated groups accounted for 0.15–0.6% of the administered dose. In rats with bile cannulae (group G2), 1.13% of the administered dose remained in the carcass (excluding the gastrointestinal contents) at sacrifice, 48 h after dosing.

The pattern of elimination of orally absorbed [U-¹⁴C]phenyl- and [2-¹⁴C]pyrimidyl-cyprodinil is shown in Table 2. The pattern of excretion was independent of sex, dose, pretreatment, and position of the radiolabel. Elimination of the radiolabel was essentially

Table 2. Elimination of [U - ^{14}C]phenyl-labelled cyprodinil and [2 - ^{14}C]pyrimidyl-labelled cyprodinil in rats (% of the administered dose)

Group	B1		C1 ^a		D1		D2		G1
	Male	Female	Male	Female	Male	Female	Male	Female	Male
Dose (mg/kg bw)	0.50	0.50	0.45	0.52	94.3	93.7	102.2	100.4	106.5
Urine									
0–8 h	32.2	26.3	25.2	28.9	25.0	23.1	14.8	19.3	24.0
8–24 h	18.3	27.6	24.6	16.7	26.1	33.2	39.6	43.0	11.4
24–48 h	1.6	2.7	1.6	2.0	1.9	2.7	5.2	4.2	—
48–168 h	0.6	1.4	0.4	0.7	0.6	0.7	1.0	1.2	—
Subtotal	52.7	58.0	51.8	48.3	53.6	59.7	60.6	67.7	35.4
Bile									
0–8 h	—	—	—	—	—	—	—	—	14.0
8–24 h	—	—	—	—	—	—	—	—	11.3
24–48 h	—	—	—	—	—	—	—	—	13.6
0–24 h	—	—	—	—	—	—	—	—	25.3
24–48 h	—	—	—	—	—	—	—	—	13.6
Subtotal	—	—	—	—	—	—	—	—	38.9
Faeces									
0–24 h	40.2	27.5	38.6	34.2	37.2	27.5	26.1	17.5	6.3
24–48 h	4.4	8.0	5.7	11.1	5.5	8.3	9.3	9.2	7.6
48–168 h	0.7	2.0	0.5	1.6	0.8	1.6	1.5	2.1	—
Subtotal	45.3	37.5	44.8	46.9	43.5	37.4	36.9	28.8	13.9
Expired air									
0–24 h	ND	ND	ND	ND	<0.1	<0.1	<0.1	<0.1	—
24–48 h	ND	ND	ND	ND	<0.1	<0.1	<0.1	<0.1	—
Subtotal	ND	ND	ND	ND	<0.1	<0.1	<0.1	<0.1	—
Cage wash									
0–168 h	0.2	1.2	0.1	0.5	0.2	0.2	0.3	0.3	1.7
Total excretion									
0–48 h	96.7	92.1	95.6	92.8	95.7	94.7	95.0	93.1	90.0
0–168 h	98.2	96.7	96.6	95.7	97.3	97.1	97.8	96.8	—
Tissue	0.21	0.37	0.15	0.33	0.35	0.50	0.40	0.60	1.13

From Thanei (1992)

^a Pretreated with unlabelled cyprodinil, 14×0.5 mg/kg bw per day

ND, Not detected

—, Not measured

complete within 48 h (92–97% of the administered dose), with 96–98% being eliminated by day 7 (168 h) after dosing. Less than 0.1% of the radiolabel was detected in exhaled air. Excretion was rapid in animals with cannulated bile ducts (group G1), with 39%, 35%, and 14% of the administered dose being excreted in the bile, urine and faeces, respectively, within 48 h. The amount of radiolabel excreted via the urine and faeces was significantly reduced in animals with cannulated bile ducts, indicating that a portion of the material excreted in the bile is reabsorbed from the intestinal tract and is eliminated via the kidneys (enterohepatic circulation) in animals without bile cannulae (Thanei, 1992).

In a separate study of absorption, distribution and depletion kinetics, male and female Tif RAIf (SPF) rats were given [U - ^{14}C]phenyl-labelled cyprodinil in polyethylene glycol 200/ethanol/water, 2:1:1 (v/v), as a single oral dose at about 0.5 mg/kg bw or about 100 mg/kg bw. The experimental design is shown in Table 3.

Table 3. Design of a study of absorption, distribution and depletion kinetics of [U - ^{14}C]phenyl-labelled cyprodinil in rats

Group	No. of animals and sex	Dose		Samples collected
		(mg/kg bw)	(kBq)	
E1	3 males	0.54	196	Blood: 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 h
	3 females	0.56		
E2	3 males	100.1	2650	Blood: 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 h
	3 females	108.5		
F1	4 × 3 males	0.52	186	Tissues: 0.5 (t_{cmax}), 2 ($t_{cmax/2}$), 10.5, 24 h
F2	3 × 3 males	93.3	2427	Tissues: 12 (t_{cmax}), 19 ($t_{cmax/2}$), 30 h
F3	4 × 3 females	0.52	185	Tissues: 1 (t_{cmax}), 2.5 ($t_{cmax/2}$), 12, 40 h
F4	3 × 3 females	101.3	2428	Tissues: 8 (t_{cmax}), 40 ($t_{cmax/2}$), 72 h

From Muller (1996)

At the lower dose, the maximum concentration of radiolabel in the blood was reached at 0.5 h in males and at 1 h in females (group E1). In females, the first peak concentration in blood (cyprodinil equivalents, 0.47 mg/kg) was six-fold higher than that in males (cyprodinil equivalents, 0.08 mg/kg). Elimination from blood followed biphasic first-order kinetics. In females, a second maximum concentration was reached at 8–12 h, indicating enterohepatic recycling. In males, the second maximum concentration was not as pronounced as in females. After the first peak, the concentration of radiolabel in blood declined rapidly; with time at which maximum concentration was reached, $t_{cmax/2}$, equal to 1 and 2 h at the lower dose for males and females, respectively. At the higher dose (group E2), the second maximum concentration was higher than the first and $t_{cmax/2}$ was 19 h and 36 h for males and females, respectively. At the lower dose (group E1), the area under the curve (AUC) was about 10-fold greater in females than in males. The residues found in the blood of animals used for investigation of tissue distribution of radiolabelled cyprodinil at the lower dose (groups F1 and F3) at various time-points were compared with the blood residues found in animals used for experiments on blood kinetics (group E1). Similar concentrations of radiolabel were found in the blood of males in group F1 and E1, while the concentrations of radiolabel in the blood of females in group E1 were approximately 17 times higher than those of females in group F3. The concentrations of radiolabel in the blood of F1 males and F3 females were similar in magnitude. Therefore, the differences in blood concentrations and the rate of elimination from the blood observed in females at the lower dose may be regarded as a fortuitous effect.

Concentrations of tissue residues were measured at t_{cmax} and $t_{cmax/2}$, and at one or two other time-points. Generally, the depletion of residues in tissues followed biphasic first-order kinetics. At the lower dose, concentrations of tissue residues initially decreased very rapidly with a half-life of <2 h, followed by a prolonged depletion phase (5–18 h). At the higher dose, the half-lives of both phases were longer in females than in males. Particularly in the slow depletion phase, the delayed depletion of blood residues affected the half-lives of residues in well-perfused organs, i.e. kidneys (248 h), lung (232 h) and spleen (182 h) (Muller, 1996).

(b) Dermal absorption

Rats

In a study of dermal absorption in rats in vivo, groups of 12 male Tif RAIf (SPF) rats received a dermal application of [2 - ^{14}C]phenyl-labelled cyprodinil, formulated as

SWITCH® 62.5 WG, at a concentration of 6 or 870 µg/cm², for a 6 h period of exposure. Four rats per dose were sacrificed immediately after 6 h, and an additional four rats per dose were sacrificed at 24 and 48 h after exposure. Skin at the application site was washed after 6 h of exposure. The lower dose (6 µg/cm²) reflected a typical concentration recommended for use in the field (final diluted spray containing 450 g of active ingredient/800 l and to be applied per hectare). The higher dose (rat skin, 1016 µg/cm²; human skin, 1376 µg/cm²) represented the highest concentration that could feasibly be applied homogeneously to the skin. [2-¹⁴C]Phenyl-labelled cyprodinil (purity, >98%) and unlabelled cyprodinil (purity, 99.9%) were mixed with the formulation ingredients (blank formulation did not contain cyprodinil). Distilled water was used as a vehicle for dermal application. On the basis of the dosing procedure described in the study, it appeared that all animals in the group receiving the lower dose had received cyprodinil at the same dose. Although there may have been small differences in the applied dose, these differences are not expected to affect overall values for dermal absorption at the lower dose, or the conclusions of the study. The application area was confined by a double "O" ring, which was glued with a cyanoacrylate adhesive to a 10 cm² previously clipped dorsal area. The test solution (100 µl) was uniformly spread over an area of 10 cm². The "O" ring was covered with permeable tape. A collar was placed over the neck area of the animal to prevent ingestion of the test substance.

Treated animals were placed in glass metabolism cages for collection urine, faeces, CO₂ and volatile substances. After the 6 h period of exposure, the cover of the "O" ring was removed and retained for analysis. The unabsorbed material was removed from the application site by washing at least three times with a mild soap solution, using cotton swabs. Finally, the moist skin was dried with cotton swabs and a fresh cover was applied to the "O"-ring for those animals to be sacrificed at 24 h and 48 h after exposure. At study termination, blood, the treated area of skin, non-treated skin, carcass, urine, faeces, skin wash, and cage wash were analysed for radiolabel.

Analysis of the skin wash showed that >98% of the radiolabel was present as parent compound at 6 h. The total recovery of radiolabel ranged from 93% to 101.5% of the applied dose. At the lower dose, the concentration of radiolabel in the blood reached a maximum (cyprodinil equivalents, 0.0037 mg/kg) at 2 h after application. Blood concentrations remained fairly constant until the end of exposure, at 6 h. Thereafter, concentrations of residue in the blood decreased to 0.0025 mg/kg at 8 h and were below the limit of determination by 24 h after application. At the higher dose, the concentration of radiolabel in the blood reached a maximum of (cyprodinil equivalents, 0.093 mg/kg) after 1 h of exposure and decreased rapidly thereafter, to below the limit of determination by 4 h.

Percutaneous absorption and excretion of cyprodinil is described in Table 4. At the lower dose, 17.3%, 21.7% and 16.6% of the applied dose was absorbed through the skin during the periods 0–6, 0–24 and 0–48 h after dosing, respectively. The amount of radiolabel in the skin wash (i.e. the dislodged dose) was 62.79%, 59.90% and 66.48% at 6, 24 and 48 h, respectively. The slightly higher dermal absorption at 24 h compared with that at 48 h may be due to poor efficacy in skin washing at 48 h. No ¹⁴CO₂ was detected in exhaled air. Only 0.02% of the applied dose was recovered in the volatile trap. The systemically absorbed dose was rapidly excreted in the urine and faeces; at 24 and 48 h, the absorbed radiolabel was excreted in the urine (9.7% and 9.0%, of the applied dose, respectively) and the faeces (8.1% and 5.9%, of the applied dose, respectively).

Table 4. Dermal absorption and excretion of cyprodinil in rats (% of applied dose)

Time-point (subgroup)	Dose ($\mu\text{g}/\text{cm}^2$)					
	Lower dose			Higher dose		
	5.9	5.9	6.9	871.1		
	t1 (6h)	t2 (24h)	t3 (48h)	t1 (6h)	t2 (24h)	t3 (48h)
Urine						
0–6 h	3.69	2.35	2.51	0.07	0.05	0.06
6–24 h	—	7.33	4.21	—	0.68	0.74
24–48 h	—	—	2.28	—	—	0.81
Subtotal	3.69	9.68	9.00	0.07	0.73	1.62
Faeces						
0–6 h	0.03	0.12	0.08	<0.01	<0.01	<0.01
6–24 h	—	7.97	3.21	—	0.30	0.35
24–48 h	—	—	2.56	—	—	0.60
Subtotal	0.03	8.09	5.85	<0.01	0.30	0.96
Expired air	ND	ND	0.02	ND	ND	ND
Cage wash	1.12	1.30	0.91	0.02	0.18	0.15
Total excretion	4.84	19.08	15.78	0.10	1.21	2.73
Residues						
Whole blood	0.09	0.03	<0.01	<0.01	<0.01	<0.01
Untreated skin	0.03	0.03	<0.01	<0.01	<0.01	<0.01
Remaining carcass	12.34	2.58	0.80	0.38	0.65	0.46
Subtotal	12.47	2.63	0.81	0.39	0.66	0.47
Systemic absorption	17.31	21.71	16.59	0.49	1.86	3.20
Treated skin	15.97	12.07	9.97	4.88	3.02	2.21
Dislodged dose	62.79	59.90	66.48	95.06	96.50	96.13
Total recovery	96.08	93.68	93.04	100.43	101.38	101.54

From Mewes (1999a)

Figures may not add up exactly, due to rounding

ND, not detected

At the higher dose, 0.5%, 1.9% and 3.2% of the applied dose was absorbed through the rat skin during the periods 0–6, 0–24 and 0–48 h after dosing, respectively. No $^{14}\text{CO}_2$ was detected in exhaled air. The systemically absorbed dose was rapidly excreted in the urine and faeces; at 24 and 48 h, the absorbed radioactivity was excreted in the urine (0.7% and 1.6% of the applied dose, respectively) and in the faeces (0.3% and 1.0%, respectively). The amount of radiolabel remaining at the site of application (treated skin) after skin washing at 6 h was 16.0% and 4.9% of the applied dose, at the lower and higher dose, respectively. The radiolabel remaining in the treated skin appears to be available for systemic absorption; at the lower dose, the amount of radiolabel at the site of application decreased to 12.1% at 24 h and to 10.0% at 48 h, compared with 16% at 6 h.

Assuming that penetration of cyprodinil through the rat skin was constant for the first 6 h of treatment, the mean penetration rate (flux) was calculated to be 0.17 and 0.71 $\mu\text{g}/\text{cm}^2$ per h for the lower and higher doses, respectively (Mewes, 1999a).

In a study of dermal absorption in vitro, epidermal membranes from rat skin and from human cadaver skin were treated with the cyprodinil formulation SWITCH®62.5 WG at a dose of 5, 1016 (rat skin) or 1376 (human skin) $\mu\text{g}/\text{cm}^2$. The lowest dose (5 $\mu\text{g}/\text{cm}^2$) approximately reflected the typical concentration recommended for use in the field (final diluted spray containing 450 g of active ingredient/800 l and applied per hectare). The higher dose (870 $\mu\text{g}/\text{cm}^2$) represented the highest concentration that could feasibly be applied homoge-

neously to the skin. [2-¹⁴C]Phenyl-labelled cyprodinil (purity, >98%) and unlabelled cyprodinil (purity, 99.9%) were mixed with the formulation ingredients and distilled water. Rat epidermal skin came from male Tif RAIf (SPF) rats and human cadaver skin came from male and female Caucasian donors. The epidermis and dermis from the frozen rat skin were separated by soaking in 2 mol/l aqueous sodium bromide solution containing 0.01% sodium azide for approximately 18 h. The epidermis and dermis were separated from the frozen human skin by hot-water treatment. The samples of epidermis from the rat and human skin were mounted on flow-through cells between donor and receptor chambers. The diffusion cells were placed on manifolds and connected to a peristaltic pump. The integrity of the epidermal membranes was checked using tritiated water. Rat epidermis with a permeability coefficient (K_p) value of $>3.5 \times 10^{-3}$ /cm per h and human epidermis with a K_p value of $>2.5 \times 10^{-3}$ /cm per h were rejected. Those cells containing membranes of acceptable K_p , according to the integrity test, received 6 μ l of the formulation; the dosing suspension was spread uniformly over a surface area of 0.64 cm² on the donor side. The donor chamber was kept open to the air. The receptor fluid was pumped through the receptor chamber. The receptor fluid used was saline during integrity testing with tritiated water and ethanol/water (1:1 v/v) during the exposure to cyprodinil. Perfusates were collected at various intervals for up to 48 h and analysed for radioactivity. At termination, the epidermal surface was rinsed with ethanol/water (1:1 w/v). The treated skin was analysed for radioactivity. The cells were disassembled and washed with ethanol/water (1:1 w/v) and the contents were analysed for radioactivity.

The nature of the radiolabelled compounds contained in the skin rinse of rat and human epidermis was analysed by the thin-layer chromatography (TLC), which indicated that >94% of the radiolabel remained as unchanged cyprodinil after 48 h. The mean recovery of radiolabel ranged from 94.1% to 108.5%. At the lower dose, 61.9%, 68.7%, and 70.3% of the applied dose penetrated the rat epidermis in 12, 24 and 48 h, respectively. At the higher dose, 3.3%, 7.6% and 16.7% of the applied dose penetrated the rat epidermis in 12, 24 and 48 h, respectively. The flux, which reflects the absorption rate under steady-state conditions, was 0.58 μ g/cm² per h for rat epidermis (steady-state conditions from about 1 to 5 h) at the lower dose. At the higher dose, the flux for rat epidermis was 4.5 μ g/cm² per h (steady-state conditions from about 4 to 48 h).

At the lower dose, 8.4%, 15.2%, and 26.5% of the applied dose penetrated the human epidermis in 12, 24 and 48 h, respectively. At the higher dose, 0.3%, 0.7%, and 1.8% of the applied dose penetrated the human epidermis in 12, 24 and 48 h, respectively. The flux, which reflects the absorption rate under steady-state conditions, was 0.03 μ g/cm² per h for human epidermis (steady-state conditions from about 2 to 30 h) at the lower dose. At the higher dose, the flux for the human epidermis was 0.60 μ g/cm² per h (steady-state conditions from about 8 to 48 h).

In conclusion, the dermal penetration of cyprodinil formulated as SWITCH® 62.5 WG was very much lower through human epidermis than through rat epidermis. The ratios of human to rat flux constants were 1:19 at a concentration of 5 μ g/cm², and 1:7.5 at the higher concentration (1016 and 1376 μ g/cm² for rat and human skin, respectively) (Mewes, 1999b).

1.2 Biotransformation: oral administration

Rats

The proposed metabolic pathways of cyprodinil in rats, goats and hens are shown in Figure 2. High-performance liquid chromatography (HPLC) analysis of rat urine identified eight different fractions (U1–U8), each accounting for 1–35% of the administered dose (Table 5). There were no qualitative differences related to sex, doses, treatment regimen, presence of bile cannulae, or type of radiolabel, but a quantitative difference between males and females was observed for metabolite fractions U2 and U6 (Table 5). The quantitative difference in metabolites in the group of animals with bile cannulae compared with other groups was caused by lower renal excretion. The similar pattern of metabolites in urine and faeces for both types of radiolabel, and no radiolabel-dependent differences in the excretion and residue data indicated that the C–N–C bridge between the phenyl and pyrimidyl rings was not cleaved. No metabolite fraction corresponded to unchanged test material.

HPLC analysis of faecal extracts from animals receiving the higher dose revealed eight metabolite fractions in extract 1. Fraction F8 corresponded to the parent compound (3–8% of the administered dose), and F6 corresponded to CGA 304076 (4-cyclopropyl-6-hydroxy-methyl-*N*-phenyl-2-pyrimidinamine). The pattern of metabolites was essentially independent of sex, dose, treatment regimen and type of radiolabel, but showed quantitative differences between the two doses (Table 6). The pattern of faecal metabolites for rats with bile cannulae (group G1) was less complex, with >90% of the administered dose corresponding to unchanged parent compound.

HPLC analysis of bile identified nine metabolite fractions, each accounting for 1–11% of the administered dose (total, 39.0%). None of the fractions corresponded to parent material, but most fractions showed similar chromatographic characteristics to those of the urine metabolite fractions (Thanei, 1992).

In another study, excreta (group D1 and D2) and bile (group G1) from Tif RAIf (SPF) rats treated with radiolabelled cyprodinil in a previous study by Thanei (1992) were used to characterize, isolate and identify metabolites of cyprodinil. The design of the study is

Table 5. Quantitative pattern of urinary metabolites in rats treated orally with radiolabelled cyprodinil (% of applied dose in metabolite fraction)

Urinary metabolite fraction	Group								
	B1		C1		D1		D2		G1
	Male	Female	Male	Female	Male	Female	Male	Female	Male
U1	3.3	2.3	ND	ND	1.9	2.5	1.3	2.5	1.4
U2	12.8	0.5	13.7	ND	13.6	0.6	13.8	0.8	11.5
U3	3.3	2.0	2.7	2.6	2.5	2.4	2.7	3.7	
U4	5.9	8.3	6.9	7.4	5.5	6.7	4.8	7.4	2.7
U5	0.8	0.5	0.6	0.7	1.2	0.7	1.3	1.2	1.3
U6	17.2	34.7	19.0	30.0	17.0	31.0	19.8	34.3	9.5
U7	3.6	2.3	3.4	2.7	5.4	5.9	8.4	8.3	6.7
U8	5.1	5.9	5.1	4.3	4.0	6.4	7.6	8.2	2.4
Total identified	52.0	56.5	51.4	47.7	51.1	56.2	59.7	66.4	35.5

From Thanei (1992)

ND, not detected

Table 6. Quantitative pattern of faecal metabolites in rats treated orally with radiolabelled cyprodinil (% of applied dose in metabolite fraction)

Faecal metabolite fraction	Group									Assignment to reference compounds
	B1		C1		D1		D2		G1	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	
F1	ND	ND	ND	ND	0.9	0.6	0.5	0.6	0.2	CGA 304076 ^a (CGA 232449) ^b
F2	4.7	3.1	ND	Traces	1.5	0.4	0.2	0.5	0.3	
F3	5.9	ND	2.3	2.7	3.6	2.4	2.3	1.8	0.3	
F4	ND	ND	ND	ND	2.8	2.1	2.3	1.5	ND	
F5	3.0	2.9	2.7	3.4	4.5	3.4	4.3	3.9	ND	
F6	6.4	5.2	11.4	11.3	11.2	10.4	8.1	4.8	ND	
F7	3.9	3.2	1.1	2.2	1.3	1.4	0.8	0.7	ND	Cyprodinil
F8	4.2	4.9	8.1	5.4	4.2	2.7	4.5	2.6	12.6	
Σ Extract 1 ^c	28.1	19.3	25.6	25.0	30.0	23.4	23.0	16.4	13.4	
Extract 2 ^d	2.8	3.0	3.8	3.5	3.2	2.4	3.4	2.6	0.1	
Residue	13.7	13.2	14.7	16.8	9.6	10.0	9.1	7.6	0.4	
Total	44.6	35.5	44.1	45.3	42.8	35.8	35.5	26.6	13.9	

From Thanei (1992)
ND, not detected
^aCGA 304076 is 4-cyclopropyl-6-methyl-2-phenylamino-pyrimidine-5-ol
^bCGA 232449 is (6-cyclopropyl-2-phenylamino-pyrimidine-4-yl)-methanol
^cΣ Extract 1 = sum of radioactivity of faecal metabolites F1–F8, from faecal samples extracted in methanol:water 4:1 (v/v)
^dExtract 2 = radioactivity in faecal sample after a second extraction of extract 1 with methanol:water 4:1 (v/v) in a soxhlet apparatus for about 20h

described in Table 1. Identification of metabolites in the urine and bile and their relative proportions are shown in Table 7; identification of faecal metabolites and their relative proportions are shown in Table 8. Eleven metabolites were isolated from urine, faeces and bile; proposed metabolic pathways in the rat are described in Figure 2. HPLC analysis of the samples of urine revealed the presence of eight metabolite fractions designated U1–U8. HPLC analysis of the samples of faeces revealed the presence of eight metabolite fractions designated F1–F8. All urinary and biliary metabolites (with the exception of 7U) were conjugated with glucuronic acid or sulfonated, and excreted. Cyprodinil was almost completely metabolized by hydroxylation of the phenyl ring (position 4) or the pyrimidine ring (position 5), followed by conjugation. An alternative pathway involved oxidation of the phenyl ring, followed by conjugation with glucuronic acid. A quantitative difference between the sexes was observed with respect to sulfonation of the major metabolite that formed 6U. The monosulfate metabolite (1U) was predominant in females, whereas equal amounts of mono- and disulfate (6U) conjugates were noted in males. Most of the significant metabolites in faeces were exocons of biliary metabolites (2U, 3U, 1G). These were assumed to be deconjugated in the intestines, partially reabsorbed into the general circulation, conjugated again, and eliminated renally. Faecal metabolite 1F is the exocon of metabolite 2U. Faecal metabolite 2F is the exocon of metabolite 3U and metabolite 1G. The major metabolic pathways of cyprodinil were not significantly influenced by dose, treatment regimen, or sex of the animal (Muller, 1992).

In a separate study, three male Tif RAIf (SPF) rats were given [2-¹⁴C]pyrimidine-labelled cyprodinil as a single dose at 100 mg/kg bw by gavage. The animals were sacrificed 12h after administration of the test substance and blood, urine, faeces, liver, kidneys and

Table 7. Identification of metabolites in the urine and bile and assignment to quantitative profiles^a (% of the administered dose in metabolite fraction)

Urine fraction	Assignment of metabolites	Corresponding bile metabolite fraction	Group				
			D1 ^b		D2 ^c		G1 ^d
			Male	Female	Male	Female	Male
U1	7U	G1	1.9	2.5	1.3	2.5	1.4
U2	6U	G2	13.6	0.6	13.8	0.8	} 11.5
U3	5U	G3	2.5	2.4	2.7	3.7	
U4	4U	G4	5.5	6.7	4.8	7.4	
U5	—	—	1.2	0.7	1.3	1.2	1.3
U6	1U	G5	17.0	31.0	19.8	34.2	9.5
U7	3U	G7	5.4	5.9	8.4	8.3	6.7
U8	2U	G8	4.0	6.4	7.6	8.2	2.4
		G6 (= 1G [80%], 2G [20%])	—	—	—	—	—
		G9	—	—	—	—	—
% of radioactivity identified			51.1	56.2	59.6	66.4	35.4

From Muller (1992)
^a See Figure 2 for identification of metabolites and metabolic pathway
^b Animals in group D1 were treated with [U-¹⁴C]phenyl-labelled cyprodinil as a single oral dose at 100 mg/kg bw and excreta were collected up to 168 h
^c Animals in group D2 were treated with [2-¹⁴C]pyrimidyl-labelled cyprodinil as a single oral dose at 100 mg/kg bw and excreta were collected up to 168 h
^d Animals in group G1 had cannulated bile ducts and were treated with [U-¹⁴C]phenyl-labelled cyprodinil as a single oral dose at 100 mg/kg bw and bile was collected up to 48 h. The value represents the amount of radiolabel in bile as a % of the administered dose

Table 8. Identification of faecal metabolites (% of the administered dose)

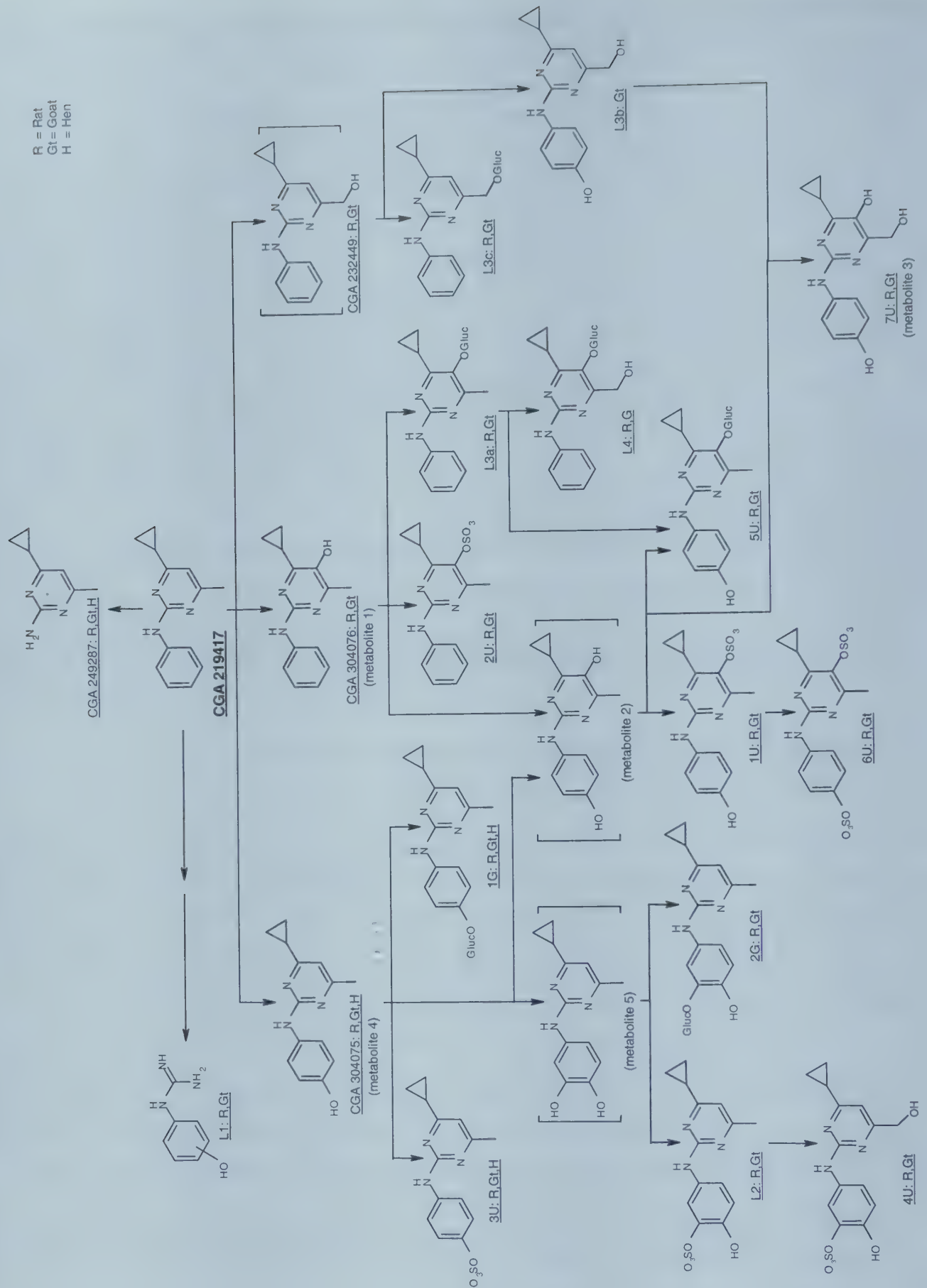
Faecal extract fraction	Assignment of metabolites	Group				
		D1		D2		G1
		Male	Female	Male	Female	Male
F1	—	0.9	0.6	0.5	0.6	0.2
F2	—	1.5	0.4	0.2	0.5	0.3
F3	—	3.6	2.4	2.3	1.8	0.3
F4	—	2.8	2.1	2.3	1.5	ND
F5	2F	4.5	3.4	4.3	3.9	ND
F6	1F	11.2	10.4	8.1	4.8	ND
F7	—	1.3	1.4	0.8	0.7	ND
F8 ^a	CGA 219417	4.2	2.7	4.5	2.6	12.6
Σ Extract 1 ^b	—	30.0	23.4	22.9	16.4	13.4
Σ Extracts & residue ^c	—	42.7	35.8	35.4	26.7	13.9

From Muller (1992)
^a Corresponds to parent compound
^b Σ Extract 1 = sum of radioactivity in fractions F1–F8, the pooled faeces from 0–48 h were extracted with 4.7 ml of methanol/g for 30 min at room temperature, with agitation. After filtration, this procedure was repeated twice with methanol/water (4:1 v/v), and all extracts were combined
^c Σ Extracts & residue = total radioactivity in extract 1 and residue (residual radiolabel in solid after extraction, i.e. nonextractable radioactivity)

residual carcass were collected. The amount of radiolabel in various tissues and excreta was determined. The amounts of metabolites in selected tissues were also determined.

The pattern of metabolites in urine was similar to that described in the previous study by Muller (1992), apart from the detection of additional metabolites CGA 249287 (4-cyclopropyl-6-methyl-pyrimidine-2-ylamine) and L1 (hydroxylated *N*-phenyl-guanidine; a

Figure 2. Proposed metabolic pathways for cyprodinil



breakdown product of the pyrimidyl ring) in the liver. Cyprodinil was found as a minor residue in liver (2.9%) and kidneys (1%). Hydroxylation in position 4 of the phenyl ring produced CGA 304075, i.e. 4-(4-cyclopropyl-6-methyl-pyrimidine-2-ylamino)-phenol; this compound was found in the liver (5.9%) and kidneys (2%), together with its sulfate (3U) and glucuronide conjugate (1G), which were also present in the urine.

CGA 304076, i.e. 4-cyclopropyl-6-methyl-2-phenylamino-pyrimidine-5-ol, was the product of hydroxylation in position 5 of the pyrimidine ring. Its sulfate conjugate (2U) and glucuronide conjugate (L3a) were found in the liver, kidney and urine. Additional hydroxylation of the glucuronide at the methyl group resulted in the minor metabolite (L4), i.e. the glucuronide of 4-cyclopropyl-6-hydroxymethyl-2-phenylamino-pyrimidine-5-ol.

Direct hydroxylation at the methyl group of cyprodinil also took place, as shown by the presence of the glucuronic acid conjugate (L3c, (6-cyclopropyl-2-phenylamino-pyrimidine-4-yl)-methanol) in the liver and urine. Hydroxylation and subsequent sulfate conjugation in position 3 of the phenol ring occurred in the dihydroxylated metabolite L2 (3-sulfate of 4-(4-cyclopropyl-6-methyl-pyrimidine-2-ylamino)-benzene-1,2-diol) and in the trihydroxylated metabolite 4U (3-sulfate of 4-(4-cyclopropyl-6-hydroxymethyl-pyrimidine-2-ylamino)-benzene-1,2-diol). These two metabolites were found in the kidney and urine.

Other dihydroxylated metabolites, dihydroxylated in positions 4 and 5 of the phenol and pyrimidine ring, respectively, were found as the sulfate (1U), bis-sulfate (6U) and glucuronide conjugate (5U) in the liver, kidney and urine. The trihydroxylated metabolite 7U (4-cyclopropyl-6-hydroxymethyl-2-(4-hydroxy-phenylamino)-pyrimidine-5-ol) was found in the liver, kidneys, and urine (Rumbeli, 1996).

Absorption, distribution, metabolism and excretion of cyprodinil administered as a single dose at 0.5 or 100 mg/kg bw by gavage was studied in male and female Tif RAIf (SPF) rats by Thanei (1992) and Muller (1992). Cyprodinil was rapidly excreted (92–97% within 48 h), irrespective of dose or sex. Approximately 53–60% and 37–45% of the administered dose was excreted within 168 h into the urine and faeces, respectively. The pattern of metabolites in urine exhibited a significant sex-related difference with respect to the major metabolite. In the urine of males, both the metabolites 1U and 6U were detected in amounts of 13–20% of the dose, while metabolite 6U was found in females only in marginal amounts (0–0.8%) (see Figure 2 for identification of metabolites). The sum of radio-label in 1U and 6U was in the same range in both sexes (30–34% and 30–35% of the administered dose for males and females, respectively). Cyprodinil is metabolized by sequential oxidation of phenyl and pyrimidinyl rings. Hydroxylation of the phenyl or the pyrimidinyl ring yields the 4-hydroxyphenyl (metabolite 4) or 5-hydroxypyrimidinyl metabolites (metabolite 1), respectively, which are excreted as sulfate conjugates (2U and 3U, respectively). A minor pathway is the further oxidation of the 4-hydroxy moiety to a 3,4-dihydroxyphenyl metabolite (metabolite 5), which is sulfated at the 3-hydroxy group (L2).

The major pathway involves hydroxylation on both rings to form a 4-hydroxyphenyl-5-hydroxypyrimidinyl metabolite (metabolite 2). Before excretion, metabolite 2 is conjugated with sulfate and, to a lesser extent, with glucuronic acid. Sulfation and glucuronidation of the 5-hydroxypyrimidinyl group, yielding 1U and 5U respectively, occurs to the same extent in males and females. However, in males 1U undergoes further sulfation at the 4-hydroxy-

phenyl group, yielding the disulfate 6U, which is not found in females. Small amounts of a trihydroxy metabolite (7U) are excreted unconjugated in males and females.

Disulfate conjugates are rarely observed in the metabolism of xenobiotics, but they are well known in the metabolism of endogenous dihydroxysteroids and dihydroxy-diarylamines, which is catalysed by steroid and phenol sulfotransferases, respectively. A disulfate conjugate was found to be a major metabolite of diphenylamine in rat urine (metabolite 2). In the sulfation of metabolite 2 in males, two distinct sulfotransferases appear to be involved. The first sulfotransferase mediates the transfer of sulfonate to the 5-hydroxypyrimidyl site. As the polarity and hydrophilicity of 1U are increased markedly in comparison to metabolite 2, the sulfation of the 4-hydroxyphenyl group is most likely to be catalysed by a second sulfotransferase having a different specificity. Since only males formed 6U, the activity of the second sulfotransferase is sex-dependent (Muller et al., 1999).

1.3 Effects on enzymes and other biochemical parameters

No information was available.

2. Toxicological studies

2.1 Acute toxicity

Data on the acute toxicity of cyprodinil are summarized in Table 9.

(a) Oral administration

Groups of five male and five female Tif RAIf (SPF) rats were given cyprodinil (purity, 99.5%) in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) as a single dose at 2000 mg/kg bw by gavage. Gross necropsy was performed at the end of a 14-day period of observation. This study complied with good laboratory practice (GLP). Rats treated with cyprodinil exhibited clinical signs such as piloerection, hunched posture, dyspnoea and reduced locomotor activity (on the day of administration in males only) after a single dose of 2000 mg/kg bw, and all treated animals recovered within 5 days. There was no mortality or significant gross pathological findings (Hartmann, 1990a).

Groups of five male and five female Tif MAG (SPF) mice were given cyprodinil (purity, 99.2%) in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) as a single dose at 5000 mg/kg bw by gavage. Gross necropsy was performed at the end of a 14-day period of observation. This study complied with GLP. Mice treated with

Table 9. Acute toxicity of cyprodinil^a

Species	Strain	Route	LD ₅₀ (mg/kg bw) or LC ₅₀ (mg/l of air)	Reference
Rat	Tif RAIf (SPF)	Oral	>2000 mg/kg bw	Hartmann (1990b)
Mouse	Tif MAG (SPF)		>5000 mg/kg bw	Winkler (1995)
Rat	Tif RAIf (SPF)	Dermal	>2000 mg/kg bw	Hartmann (1990a)
Rat	Tif RAIf (SPF)	Inhalation	>1200 mg/m ³	Hartmann (1991)
Rabbit	New Zealand white	Dermal irritation	Not an irritant	Schneider (1990a)
Rabbit	New Zealand white	Ocular irritation	Not an irritant	Schneider (1990b)
Guinea-pig	Pirbright white	Skin sensitization: maximization test	Sensitizer	Winkler (1996a)

^aTechnical grade cyprodinil of >99% purity was used in all studies

cyprodinil exhibited clinical signs such as piloerection and hunched posture, which occurred only in the first 5 h after dosing. These signs were not considered to be compound-related effects. No mortality occurred and no gross abnormalities were observed at necropsy (Winkler, 1995).

(b) *Dermal application*

Groups of five male and five female young adult Tif RAIf (SPF) albino rats received a dermal application of cyprodinil (purity, 99.5%) at a dose of 2000 mg/kg bw (limit dose) for 24 h. The test substance, suspended in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v), was applied to approximately 10% of the total surface area of the body. Animals were observed for clinical signs and mortality for up to 14 days after dosing. This study complied with GLP. Rats treated with cyprodinil exhibited clinical signs such as piloerection, abnormal body position, and dyspnoea after a single dermal application of 2000 mg/kg bw; all treated animals recovered within 5 days. No mortality was observed in the 14-day period of observation. No gross abnormalities were observed at necropsy (Hartman, 1990b).

(c) *Exposure by inhalation*

Five male and five female young adult Tif RAIf (SPF) rats were exposed by nose-only inhalation to cyprodinil (purity, 99.5%) at a dose of 1.20 mg/l (the highest attainable concentration) for 4 h. A control group of five males and five females were exposed to filtered humidified air only. Animals were observed for clinical signs and mortality for up to 14 days after exposure. This study complied with GLP. Rats exposed to cyprodinil via nose-only inhalation exhibited clinical signs such as piloerection and dyspnea after a single exposure of 1.2 mg/l for 4 h; all treated animals recovered within 5 days. No mortality was observed. No gross abnormalities were observed at necropsy (Hartmann, 1991).

(d) *Dermal and ocular irritation*

In a study of primary dermal irritation, three female Chbb:NWZ rabbits received an application of 0.5 g of cyprodinil (purity, 99.5%), moistened with carboxymethylcellulose (0.5% w/v) in aqueous polysorbate 80 (0.1% w/v), for 4 h. A gauze patch (surface area, approximately 12–16 cm²) containing the test article was applied to the right flank of the each animal. A control gauze patch was applied to the contralateral flank. This study complied with GLP. No skin reactions were observed at 24 and 72 h after application. No mortality was observed (Schneider, 1990a).

In a study of primary ocular irritation, three male Chbb:NWZ rabbits received an instillation of 0.1 ml (40 mg) of undiluted cyprodinil (purity, 99.5%) into the conjunctival sac of the left eye. The eyes were observed for 72 h after instillation and scored for irritation at 1, 24, 48, and 72 h according to the OECD scoring system. This study complied with GLP. Slight to moderate conjunctival redness and mild chemosis were observed at 1 h, and mild redness in one animal after 24 h. All treated eyes recovered within 48 h after instillation (Schneider, 1990b).

(e) *Dermal sensitization*

In a study of dermal sensitization using the maximization method of Magnusson and Kligman, cyprodinil (purity, 99.2%) was given to 10 male and 10 female 10-week-old Tif DHP (Pirbright white) guinea-pigs. An additional five animals of each sex were used as

controls and received adjuvant, vehicle, or challenge treatment only. After the challenge, 9 out of 20 treated animals (45%) exhibited very slight or well-defined erythema (Draize score of 1 or 2) without oedema 24 h after Hilltop chamber and dressing removal. At 48 h, very slight erythema without oedema persisted in 6 out of 20 treated animals (30%). Two of these animals (both females) had scaling erythema. No other treated animal and no control animal exhibited any dermal irritation caused by the challenge application. Data from positive controls indicated an appropriate positive response. This study complied with GLP (Winkler, 1996a).

2.2 *Short-term studies of toxicity*

Mice

In a 3-month study of toxicity, groups of 10 male and 10 female Tif MAGf (SPF) albino mice were fed diets containing cyprodinil (purity, 99.5%) at a concentration of 0, 500, 2000 or 6000 mg/kg (equal to 0, 73.32, 257.3 or 848.6 mg/kg bw per day in males and 0, 102.5, 349.2 or 1121.0 mg/kg per day in females). Animals were examined at least once daily for morbidity and mortality, and body weights and food consumption were recorded weekly. Haematological parameters were measured for all surviving animals at each dose at study termination. All animals that survived to study termination were subjected to detailed necropsy. Organ weights were recorded and histological examinations were performed. This study complied with GLP.

There were no treatment-related effects on mortality, clinical signs or haematological parameters. There was no clear treatment-related effect on body weight, and there were no consistent differences in body-weight gains between control and treated animals at various times throughout the study. However, a slight trend towards reduced body-weight gain was observed at the highest dose. There was no treatment-related effect on food consumption or food consumption efficiency. Values for overall food consumption in treated animals were within 5% of those for controls.

There were dose-related increases in mean absolute liver weights, mean liver to body weight ratios, and mean liver to brain weight ratios in males at the intermediate and highest doses, relative to controls; these were statistically significant at the highest dose. Mean absolute and relative spleen weights were significantly greater in females at the intermediate and highest doses, and mean absolute spleen weight was significantly greater in males at the highest dose. However, there was no dose-response relationship for absolute spleen weights and no haematological or histopathological findings to support a toxicologically significant effect. Absolute and relative thyroid weights showed a dose-related decrease in males; however, values for these parameters were slightly increased in females at the highest dose. In the absence of histopathological findings, thyroid changes were not considered to be toxicologically significant.

There were no macroscopic findings in control or treated animals. Moderate multifocal single cell hepatocyte necrosis was observed throughout the liver parenchyma in 3 out of 10 males in each group receiving the intermediate or highest dose, and glycogen depletion was noted in 7 and 10 out of 10 females at the intermediate and highest dose, respectively. No other findings were considered to be related to treatment.

The no-observed-effect level (NOEL) was 500 mg/kg (equal to 73.3 mg/kg bw per day in males, 103 mg/kg bw per day in females) on the basis of multifocal single cell hepato-

cyte necrosis at 2000 and 6000 mg/kg in males and glycogen depletion at 2000 and 6000 mg/kg in females. Single cell necrosis was moderate and multifocal in all affected animals, the lesion being observed throughout the liver parenchyma. It should, however, be noted that no dose–response relationship was observed in this study, and there was no clear treatment-related effect on liver necrosis in the 18-month study in mice. As stated in the study report, glycogen depletion was indicative of physiological stress, starvation, or an underlying pathological condition, particularly in young animals, in which glycogen in the cytoplasm is essential for liver metabolism (Fankhauser, 1991a).

Rats

In a 28-day range-finding study, groups of 10 male and 10 female Tif RAIf (SPF) rats were given cyprodinil (purity, 99.5%) in carboxymethylcellulose (0.5% w/v)/Tween 80 (0.1% w/v) in distilled water, at a dose of 0, 10, 100 or 1000 mg/kg bw per day by gavage, once daily for 28 days. Animals were observed twice daily on working days and once at weekends for mortality, and daily for clinical signs of toxicity. Individual body weight, food and water consumption were recorded weekly. Ophthalmoscopic examinations of animals in the control groups and the groups receiving a dose of 1000 mg/kg bw per day were conducted 5 days before and 23 days after the initiation of treatment. Haematological and clinical chemistry tests and necropsy were performed at the end of the study period. Urine analysis was not conducted in this study. At the end of the study, gross necropsies were performed and selected organs were removed, weighed and histopathological examination performed. This study complied with GLP.

No treatment-related clinical signs of toxicity or deaths occurred during the study. Mean body-weight gains for male rats at 1000 mg/kg bw were 22% lower ($p < 0.05$) than those for controls after 2 weeks of treatment. Between weeks 2 and 4, these treated rats gained more weight than the controls, so the total body-weight gain for male rats at 1000 mg/kg bw at the termination of the study was only 9% lower than that of the controls. Body-weight gains for female rats at 1000 mg/kg bw were 19% and 15% lower than those of the controls at weeks 2 and 4, respectively; these differences were not statistically significant. The body weights and body-weight gains of males and females at 10 and 100 mg/kg bw were not significantly different from those of the controls.

During the first week of treatment, food consumption (g of food/kg bw per day) in males and females at 1000 mg/kg bw was 30% lower than food consumption in the same animals before initiation of treatment and 8–10% lower than that in the control groups during the first week of treatment. During the remainder of the study, food consumption (g of food/kg bw per day) in animals in the groups receiving the highest dose was similar to that in the controls. Food consumption at 10 or 100 mg/kg was similar to that in the controls.

Water consumption (g of water/animal) at 100 (males only) and 1000 mg/kg bw was slightly higher than that in the controls.

No treatment-related ophthalmological abnormalities were noted during the study. Urine analysis was not conducted in this study.

Rats in the groups receiving a dose of 100 and 1000 mg/kg had higher leukocyte counts and lower mean corpuscular haemoglobin (MCH) values compared with those of the

controls. At 1000 mg/kg, prothrombin times were had significantly faster (up to 25–28%) than those of the controls. The slight increase in leukocyte count and minimal decrease in MCH values at 100 mg/kg bw per day were within the range of values for historical controls and normal biological variation and were considered not to be toxicologically relevant.

Rats treated with a dose of 1000 mg/kg bw had higher concentrations of blood protein (11% higher), albumin (7–10% higher), globulin (12–18% higher), total bilirubin (21–29% higher), cholesterol (56–71% higher), and phospholipid (41–51% higher) than the control animals. At the highest dose, slightly elevated serum activities were observed for alanine aminotransferase and alkaline phosphatase. No other treatment-related differences existed between the treated and the control rats; all other observed differences in blood chemistry remained within the expected biological ranges for this strain of rat.

Increased absolute and relative liver weights were observed at 100 mg/kg bw per day (10–17% greater than controls) and 1000 mg/kg bw per day (27–54% greater than controls); the increases were statistically significant for both sexes at 1000 mg/kg bw per day. Increased absolute thyroid weights (7–16%) and relative thyroid weights (12–23%) were observed at 1000 mg/kg bw per day. These differences were not statistically significant.

No toxicological relevance was attributed to slight variations in thymus weight since the decrease was not dose-related, and was statistically significant only for treated females.

Enlargement of the liver was noted in one female at 100 mg/kg bw per day, and in nearly all animals at 1000 mg/kg bw per day. “Minimal” hypertrophy of liver hepatocytes was noted at 100 (females only) and 1000 mg/kg bw per day. Slightly increased liver weight, associated with a minimal hepatocellular hypertrophy, was considered to be an adaptive response in the liver. Minimal to moderate hypertrophy of the follicular epithelium of the thyroid gland was noted in almost all animals at 1000 mg/kg bw per day. No abnormalities were observed in the thymus of the treated rats. All other abnormalities occurred randomly and sporadically in all groups. No neoplastic tissue was observed in rats in the treated or control groups. Although the NOEL identified by the study author was 10 mg/kg bw per day, this reviewer considered that the no-observed-adverse-effect level (NOAEL) was 100 mg/kg bw per day. The lowest-observed-adverse-effect level (LOAEL) was 1000 mg/kg bw per day for rats, on the basis of slightly decreased body weight, decreased food consumption, increased liver weights, changes in haematological and clinical chemistry parameters, abnormalities in liver morphology and hypertrophy of the follicular epithelium of the thyroid glands (Fankhauser, 1991c).

In a 28-day study of dermal toxicity, groups of five male and five female Tif RAIf (SPF) rats received repeated dermal applications of cyprodinil (purity, 99.5%) at a dose of 0, 5, 25, 125 or 1000 mg/kg bw per day, for 6 h per day, 5 days per week over 28 days. The fur of each animal was clipped from the dorsal area of the trunk over an area of at least 10% of the body surface shortly before the first application and weekly thereafter. The test substance was suspended in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v). The test article/vehicle suspension was applied to the clipped area and held in place under an occlusive dressing. Rats in the control group were exposed to the vehicle only, using the same procedure. Animals were observed daily for mortality, signs of toxicity, and the presence of dermal irritation. The animals were also examined for signs of local skin irritation approximately 17 h after removing the gauze patches and were evaluated

according to the method of Draize. Animals were weighed before the start of treatment, then weekly, to ensure accurate dosing. Body weights were recorded weekly. Food consumption for each cage of animals (five rats per cage) was determined weekly. Haematological and clinical chemistry parameters were measured for surviving animals at study termination. All animals were sacrificed on schedule and subjected to gross pathological examination. Selected tissues were collected for weighing and histological examination. This study complied with GLP.

No animals died or had to be killed during the course of the study. Clinical signs were observed in females at > 25 mg/kgbw per day and in males at 1000 mg/kgbw per day. Pilo-erection was seen in: 2 out of 5 females at 25 mg/kgbw per day primarily during week 4; four out of five females at 125 mg/kgbw per day during weeks 3 and 4; and in males (four out of five) and females (five out of five) at 1000 mg/kgbw per day during weeks 2–4. Hunched posture was seen in two out of five females at 125 mg/kgbw per day during weeks 3 and 4, and in four out of five females at 1000 mg/kgbw per day during week 4. Dyspnoea was observed in two out of five males and two out of five females at 1000 mg/kgbw per day during weeks 2 and/or 3. No local skin irritation or other differences in clinical signs were observed between the treated and control groups.

Body weights or body-weight gains for both sexes in all of the treated groups were not significantly different from those of the controls. Reduced food consumption (g/animal per week) in males at 1000 mg/kgbw per day during week 4 was statistically significant ($p < 0.05$) compared with that in the controls, and may have been treatment-related. No other variations in food consumption appeared to be treatment-related for any of the treated groups. Reduced food consumption in males at 5, 25, and 125 mg/kgbw per day during weeks 1, 3, and 4 was not concentration-related, although it was statistically significant ($p < 0.05$) for males at 5 and 25 mg/kg during week 3 compared with that in the controls. Decreased food consumption ratios for males at 5, 25, and 1000 mg/kgbw during weeks 3 and 4 compared with that for the controls did not appear to be concentration-related, since food consumption ratios for males at 125 mg/kgbw per day were comparable with those of the controls. Food consumption by females in all treated groups was comparable to that of the controls. Food consumption ratios for females in the treated and control groups varied throughout the study.

One female from the group receiving a dose of 1000 mg/kgbw per day exhibited anaemia associated with an increased number of reticulocytes. The toxicological relevance of this finding is considered to be equivocal, since only one animal was affected at this, the highest, dose.

No other differences in haematology were observed between any of the treated groups and the control groups. No differences in clinical blood chemistry parameters attributable to treatment were observed for any of the treated groups. Differences in clinical chemistry parameters were observed, but they were within the normal biological ranges for this strain of rat and were attributed to normal variation, although they were statistically significant ($p < 0.05$) compared with those for the controls.

No differences in absolute or relative organ weights were observed between any of the treated and control groups, except for a statistically significant ($p < 0.05$) increase in mean relative testes weight for males at 5 mg/kgbw per day. No differences in gross pathol-

ogy were observed between any of the treatment and control groups. Comparable numbers of isolated macroscopic findings occurred in all treated and control groups, and were similar to those occurring spontaneously in the colony from which the animals were selected. No neoplastic alterations attributable to treatment occurred in animals in any of the treated groups. The incidence, distribution, and morphological appearance of microscopic alterations that occurred at the dermal test site were similar in the control and treated rats, and were considered to be caused by the application procedure. All other changes were reported to be common to the colony from which the test animals were selected. No neoplastic tissue was observed in the treated or control rats.

The pattern of toxicity seen in this study appears to be more severe than that observed in the 28-day study of rats treated by gavage. Moreover, there appear to be more severe effects in females than in males; this was not apparent in other toxicological studies. The reason for these differences is not clear on consideration of the available data on dermal absorption, and other relevant data. Therefore, the results of this study should be used with caution in any risk assessment. The NOEL was given as 5 mg/kg bw per day by the study author. This reviewer considered the NOAEL to be 25 mg/kg bw per day for females and 125 mg/kg bw per day for males. The LOAEL was 125 mg/kg bw per day for females and 1000 mg/kg bw per day for males, on the basis of alterations in clinical signs (hunched posture and/or piloerection) (Hagemann, 1991).

In a short-term study of toxicity, groups of 20 male and 20 female Tif RAIf (SPF) rats were given diets containing cyprodinil at a concentration of 0 or 12 000 mg/kg (equal to 0 or 810 mg/kg bw per day for males and 0 or 803 mg/kg bw per day for females), and groups of 10 male and 10 female rats were given diets containing cyprodinil at a concentration of 50, 300, or 2000 mg/kg (equal to 3.14, 19.0, or 134 mg/kg bw per day, respectively, for males, and 3.24, 19.3, or 137 mg/kg bw per day for females) for 90 days. All animals were sacrificed after 90 days, except for 10 rats of each sex in the control group and in the group receiving cyprodinil at 12 000 mg/kg, which were sacrificed after a 4-week period of recovery.

Animals were inspected at least once each day for signs of toxicity and mortality. Animals were weighed before the start of treatment, then once per week for the duration of the study. Food and water consumption for each cage of animals (five rats per cage) was determined weekly. Mean food consumption was reported as g of food/animal per day and the food consumption ratio (g of food/kg bw per day) was calculated as an indicator of the food consumption efficiency. Food conversion efficiency was not determined. Ophthalmoscopic examinations of animals in the control group and the group receiving cyprodinil at 12 000 mg/kg were conducted 4 days before and 88 days after the start of treatment, and at the end of the 4-week period of recovery (day 115). For evaluation of clinical chemistry and haematological parameters, blood was collected from the orbital sinus of all animals surviving to termination of the study on the morning following overnight fasting. Urine analysis was performed on samples collected from fasted animals held overnight in special metabolism cages at the termination of the study. All animals that died and those that were sacrificed on schedule were subjected to gross pathological examination; selected organs were weighed and tissues were collected for histological examination. This study complied with GLP.

There were no treatment-related effects on mortality, clinical signs, urine analysis or upon ophthalmological examination.

At 12000 mg/kg, mean body weights of male and female rats were 13–17% lower ($p < 0.01$) than those of controls each week during the treatment period, and body-weight gains were 20–26% lower than those of controls at termination of the study. At the end of the recovery period, the treated rats weighed only 11–12% less than the controls, indicating some recovery of body weights. Body weights and body-weight gains of animals in other treated groups were affected to a minimal degree.

The food consumption ratio (g of food/kgbw per day) for males and females at 12000 mg/kg was 20–28% lower than that for the controls and other treated groups during the first week of the study only, indicating a palatability problem. During weeks 2–13 of treatment, food consumption by the control group and all treated groups was similar. During the first week of the 4-week period of recovery (for rats at 0 and 12000 mg/kg only), male and female rats at 12000 mg/kg consumed 36–37% more food than did the controls. For the entire 4-week period following treatment, treated rats consumed an average of 25–29% more food than the controls.

Water consumption (g of water/animal per week) for males and females at 12000 mg/kg was lower than that for the controls and other treated groups during the study.

All differences in haematology parameters that were observed between the control and treatment groups remained within the expected biological ranges for this strain of rat. Differences that carried statistical significance and may have been treatment-related included increases in haemoglobin concentration, erythrocyte volume fraction, and leukocyte count. At 12000 mg/kg, male rats had lower haemoglobin and erythrocyte volume fraction values than the controls, and male and female rats had leukocyte counts that were 19% and 56% higher, respectively, than those of the controls. At 300, 2000, or 12000 mg/kg, male rats also had prolonged prothrombin times in comparison with those of controls. In contrast, while females at 12000 mg/kg had prolonged prothrombin times compared with those of the controls, females at 300 mg/kg or 2000 mg/kg had reduced prothrombin times compared with the controls. The prolongation in prothrombin time seen at 300 mg/kg and 2000 mg/kg was small compared with that in concurrent controls and no clear trend was seen in either sex. Therefore, no toxicological significance is attributed to the variations in prothrombin time at 300 mg/kg and 2000 mg/kg. All haematological differences observed between the control group and the group treated with cyprodinil at 12000 mg/kg disappeared by the end of the 4-week period of recovery.

Cyprodinil had a significant ($p < 0.01$ or 0.05) effect on the clinical blood chemistry of male rats at 2000 or 12000 mg/kg, and of female rats in all treated groups. Cholesterol concentrations were higher in females at 50 (18%) and 300 (20%) mg/kg, and in males and females at 2000 (37–48%) and 12000 (85–105%) mg/kg. Phospholipid concentrations were higher in females at 50 (18%) and 300 (23%) mg/kg, and in males and females at 2000 (36%) and 12000 (71–90%) mg/kg. The slightly higher cholesterol and phospholipid concentrations measured in females at 50 and 300 mg/kg were not considered to be toxicologically relevant due to the marginal degree of increase (1.2-fold), and because values were mainly within the range of values for the concurrent control group. In addition, the 2-year study of carcinogenicity in rats did not show a treatment-related increase in blood cholesterol and phospholipid concentrations at a dose of up to 2000 mg/kg (at week 105, terminal measurements). Protein and globulin concentrations were each up to 7% higher in males at 2000 and 12000 mg/kg. In males at 12000 mg/kg, albumin concentrations were 6% higher and glucose concentrations were 11% lower than those of controls.

All other differences in blood chemistry that were observed remained within the expected biological ranges for this strain of rat. Statistically significant treatment-related increases were observed in alkaline phosphatase activity in males at 2000mg/kg and in males and female at 12000mg/kg, and in gamma-glutamyl transpeptidase activity in males and females at 12000mg/kg. Other parameters that were statistically different from controls but that did not appear to be related to treatment were aspartate aminotransferase activity (increased in males at 50 and 300mg/kg, decreased in females at 12000mg/kg), alanine aminotransferase activity (increased in males at 300, 2000, and 12000mg/kg), blood urea (decreased in males at 2000mg/kg and in females at 12000mg/kg), creatinine (increased in females at 300 and 2000mg/kg), total bilirubin (increased in males at 12000mg/kg), triglycerides (decreased in males at 12000mg/kg and increased in females at 300 and 2000mg/kg), calcium (increased in males at 2000 and 12000mg/kg, and in females at 50 and 12000mg/kg), and phosphate (increased in males at 12000mg/kg).

After 4 weeks of recovery, the blood chemistry parameters that had been affected by treatment at 12000mg/kg were similar to those of the control group, with the exception of alkaline phosphatase activity in males and cholesterol and phospholipid concentrations in females. Alkaline phosphatase activity in males at 12000mg/kg remained 24% greater than that in the control group after treatment. At 12000mg/kg, cholesterol concentrations in females decreased from 85% greater than the control group (after treatment) to 22% greater (after recovery), and phospholipid concentrations decreased from 71% to 15% greater during the same period.

Cyprodinil affected the relative weights of the liver and thyroid of rats at 2000 and 12000mg/kg, and the relative weights of the kidneys of rats at 12000mg/kg. Relative liver weights were 13–15% and 27–37% higher at 2000 and 12000mg/kg, respectively; the increases were statistically significant for males at 12000mg/kg and for females at 2000 and 12000mg/kg. The relative weights of the thyroid glands of male rats at 2000 and 12000mg/kg were 22% and 38% higher, respectively, than those of the controls. The relative weights of the thyroid glands of female rats at 12000mg/kg were 42% higher than those of the controls. The relative weights of the adrenals of males at 12000mg/kg were 22% heavier than those of controls (0.201 g versus 0.164 g); however, this difference was not statistically significant. The relative weights of the kidneys of male rats at 12000mg/kg were 14% higher than those of rats in the control group. Decreases in the absolute mean weight of the hearts, adrenals, and ovaries of female rats at 12000mg/kg, compared with that of the controls, were statistically significant, but of no apparent biological significance. No other differences were observed between rats in the treated and control groups.

After 4 weeks of recovery, the relative weights of the liver and thyroid glands of males at 12000mg/kg were 9% and 63% greater, respectively, than those of the control group. The relative weights of organs in females at 12000mg/kg and in the control groups were similar after recovery.

Tissue discoloration and all other abnormalities occurred randomly and sporadically in all groups. Histopathological changes were observed in the liver, pituitary gland, thyroid, adrenal cortex, and kidneys of treated males, and in the liver, pituitary gland, and kidneys of treated females (Table 10). Liver abnormalities, including hepatocyte hypertrophy, hepatocyte necrosis, and the presence of “sharply demarcated membranous structures containing a vacuolated eosinophilic material” (“cytoplasmic inclusion bodies”), were observed in both sexes at 2000 and 12000mg/kg. “Minimal” to “moderate” hypertrophy of hepatocytes

Table 10. Incidence of pathological findings in a 90-day study of toxicity in rats given diets containing cyprodinil

		Dietary concentration (mg/kg)									
		0		50		300		2000		12 000	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<i>Liver hepatocytes</i>											
Hypertrophy											
S1		1/10	0	1/10	0	4/10	0	9/10	6	10/10	8/10
S2		0	0	—	—	—	—	—	—	2/10	0/10
Necrosis											
S1		0	1/10	1/10	0	2/10	0	10/10	4	8/10	7/10
S2		1/10	0	—	—	—	—	—	—	1/10	0
Inclusion bodies											
S1		0	1/10	0	0	0	0	3/10	3/10	9/10	1/10
S2		0	0	—	—	—	—	—	—	2/10	0
<i>Kidney</i>											
Chronic tubular lesion											
S1		0	2/10	0	2/10	3/10	0	5/10	5/10	8/10	8/10
S2		2/10	0	—	—	—	—	—	—	8/10	6/10
Nephrocalcinosis											
S1		0	10/10	0	9/10	1/10	9/10	0	10/10	5/10	10/10
S2		0	7/10	—	—	—	—	—	—	1/10	7/10
<i>Thyroid follicular epithelium</i>											
Hypertrophy											
S1		0	0	1/10	0	4/10	0	10/10	1/10	9/10	6/10
S2		1/10	0	—	—	—	—	—	—	0	0
<i>Pituitary cell</i>											
Hypertrophy											
S1		0	0	0	0	4/10	0	10/10	2/10	8/10	4/10
S2		0	0	—	—	—	—	—	—	3/10	0

S1 = rats sacrificed at week 14; S2 = rats sacrificed at week 18 (after 4-week period of recovery)—only rats at 0 and 12 000 mg/kg

was noted in 4/10 males at 300 mg/kg, 9/10 males and 6/10 females at 2000 mg/kg, and 10/10 males and 8/10 females at 12 000 mg/kg. Monocellular foci of necrotic hepatocytes were observed in 2/10 males at 300 mg/kg, 10/10 males and 4/10 females at 2000 mg/kg, and in 8/10 males and 7/10 females at 12 000 mg/kg. The study author stated that, “1/10 male animals in both groups 4 and 5 (2000 mg/kg and 12 000 mg/kg) showed a recent necrosis of a distinct group of hepatocytes.” Cytoplasmic inclusion bodies, usually present in groups and located primarily in the hypertrophic cells attached to the periportal tract of the liver lobules, were noted in 3/10 males and 3/10 females at 2000 mg/kg, and 9/10 males and 1/10 females at 12 000 mg/kg.

Enlarged (hypertrophic) pituitary cells in the adenohypophysis were observed in 4/10 males at 300 mg/kg, 10/10 males and 2/10 females at 2000 mg/kg, and 8/10 males and 4/10 females at 12 000 mg/kg. This abnormality was observed in both single and small clusters of cells.

“Minimal” to “moderate” hypertrophy of the follicular epithelia of the thyroid was noted in 4/10 males at 300 mg/kg, 10/10 at 2000 mg/kg, and 9/10 at 12 000 mg/kg. No thyroid abnormalities were observed in female rats in any treated group.

“Minimal” increases in the number of fat vesicles in the adrenal cortex were noted in 6 out of 10 males at 12 000 mg/kg.

Kidney abnormalities were observed in male and female rats at 300, 2000, and 12 000 mg/kg. Nephrocalcinosis was noted in 1/10 males at 300 mg/kg and 5/10 males at 12 000 mg/kg, but not at 2000 mg/kg; in contrast, nephrocalcinosis was noted in the majority (90–100%) of females in the control group and in all treated groups. Vacuolization of the epithelium of the proximal convoluted tubules was observed in 1/10, 1/10, and 2/10 male rats at 300, 2000, and 12 000 mg/kg, respectively. Chronic tubular lesions were increased in male rats at 300, 2000, and 12 000 mg/kg (0, 0, 30, 50, 80%, in controls, 50, 300, or 2000 and 12 000 mg/kg, respectively) and in females only at 2000 and 12 000 mg/kg (20%, 20%, 0%, 50% and 80% in control, 50, 300, 2000 and 12 000 mg/kg, respectively).

All other abnormalities occurred randomly and sporadically in all groups after treatment.

With the exception of chronic tubular lesions in the kidneys, the prevalence of abnormalities was reduced after a 4-week period of recovery in rats that had received cyprodinil at a concentration of 12 000 mg/kg in the diet. After the period of recovery in males at 12 000 mg/kg, the following observations were made: hepatocyte hypertrophy, 2/10 animals; hepatocyte necrosis, 1/10 animals; cytoplasmic inclusion bodies in liver cells, 2/10 animals; hypertrophy of pituitary cells, 3/10 animals; and chronic tubular lesions in the kidneys, 8/10 animals. No abnormalities were observed in the thyroid, while the adrenal cortex was not examined. After recovery, females receiving cyprodinil at 12 000 mg/kg were examined only for chronic tubular lesions in the kidneys; these abnormalities were observed in 6/10 animals.

No neoplastic changes were observed in rats in treated or control groups. At 50 mg/kg, changes were not considered to be toxicologically significant and a LOAEL was not identified. At 300 mg/kg, increased incidences of hepatocyte hypertrophy, necrosis, cytoplasmic inclusion bodies, chronic tubular lesions in the kidneys, nephrocalcinosis, cytoplasmic vacuolization in proximal tubules, thyroid epithelium hypertrophy and pituitary hypertrophy were seen in males. A slightly increased incidence of minimal hypertrophy of liver hepatocytes, pituitary cells, and thyroid follicular epithelium was considered to be treatment related. However, these effects were not seen in the same strain of rats in the 90-day study of neurotoxicity at a concentration of up to 800 mg/kg, nor in the 2-year study of carcinogenicity at a concentration of up to 1000 mg/kg. Therefore, the LOAEL was set at 2000 mg/kg (equal to 134 and 137 mg/kg bw per day in males and females respectively) for rats, on the basis of effects on clinical chemistry, increased relative liver weight (females), increased absolute and relative thyroid weight (males), and histopathological findings in liver, kidneys, thyroid and pituitary. The NOAEL was 300 mg/kg (equal to 19 and 19.4 mg/kg bw per day in males and females respectively). The NOEL was 50 mg/kg, equal to a mean daily intake of 3.14 mg/kg bw per day (Fankhauser, 1991b).

Dogs

In a 90-day study of toxicity, groups of four male and four female beagle dogs were fed diets containing cyprodinil (purity, 99.5%) at a concentration of 0, 200, 1500, 7000 or 20 000 mg/kg (equal to 0, 6.07, 45.87, 210.33 or 559.66 mg/kg bw per day for males and 0, 6.79, 52.75, 231.93 or 580.95 mg/kg bw per day for females). Animals were examined at

least once daily for signs of morbidity and mortality, body weights were recorded weekly, and food consumption was recorded daily and reported as a weekly mean. Eye examination (external inspection; lens, iris, and fundus examination; pupillary reflex; third eyelid inspection) was performed before the start of treatment and at termination (week 13). Haematology, blood chemistry, and urine analysis were carried out on all animals before the start of treatment, at week 7, and at week 13. This study complied with GLP.

There were no effects on mortality, ophthalmology, or clinical chemistry parameters. Treatment-related effects included vomiting in all females receiving the highest dose during the first 3 days of treatment. No other relevant clinical symptoms were noted.

Mean body-weight gains were consistently lower in males and females receiving the highest dose relative to those of controls throughout the study period. All animals receiving the highest dose exhibited weight loss during the first 2–3 weeks of the study. At the highest dose, both sexes showed clearly depressed body-weight gains, with values being 36% (males) and 24% (females) those of control animals. A slight weight reduction was also noted in females at 7000 mg/kg after the first week of treatment; however, no differences relative to controls were noted in this group or in groups at lower doses throughout the remainder of the study.

Mean food consumption was consistently lower in animals at the highest dose, relative to controls, throughout the period of treatment; this was considered to be treatment-related. The most prominent reduction in food consumption occurred after the first week of treatment. The only difference in haematological parameters measured at weeks 7 and 13 was an increase in the mean platelet count of females at the highest dose relative to that of the controls. In the absence of any associated histopathological findings, this was not considered to be toxicologically significant.

Examination of data on mean and individual organ weights gave no clear indication of any treatment-related effect. Slight increases in relative spleen, liver, kidney, and adrenal weights were observed, but in the absence of any histopathological observations or a clear dose–response relationship, these findings were not considered to be toxicologically significant. Histopathological examinations did not reveal any treatment-related changes.

The NOAEL was 7000 mg/kg (210 mg/kg bw per day in males, 232 mg/kg bw per day in females) on the basis of lower body-weight gains and decreased food consumption in males and females at the highest dose. Lower body-weight gain and reduced food consumption in females at 7000 mg/kg was observed after the first week of treatment only, after which an adjustment was made to the palatability of the food and these effects were, therefore, not considered to be adverse. The LOAEL was 20000 mg/kg (560 and 581 mg/kg bw per day in males and females respectively). The NOEL was 1500 mg/kg, equal to 45.9 mg/kg bw per day for males and 52.8 mg/kg bw per day for females (Altmann, 1991).

In a one-year study of toxicity, groups of four male and four female beagle dogs were fed diets containing cyprodinil (purity, 99.6%) at a concentration of 0, 25, 250, 2500 or 15000 mg/kg (equal to 0, 0.72, 6.87, 65.63 or 449.25 for males and 0, 0.76, 6.80, 67.99 or 446.37 mg/kg bw per day for females). Animals were examined at least once daily for signs of morbidity and mortality, body weights were recorded weekly, and food consumption was recorded daily and reported as a weekly mean. Eye examination (external inspection; lens, iris, and fundus examination; pupillary reflex; third eyelid inspection) was performed before

the start of treatment and at termination (week 52). Haematology, blood chemistry, and urine analysis were carried out on all animals before the start of treatment, and at weeks 13, 26 and 52. All animals survived to study termination and were subjected to detailed necropsy. Organ weights were recorded and full histological examinations were performed. This study complied with GLP.

There were no treatment-related effects on mortality, clinical signs, ophthalmology, urine analysis, haematology or clinical chemistry parameters. Any findings that were statistically significant were sporadic.

Mean body-weight gain was reduced in both sexes at 15 000 mg/kg (69% and 62% of that of the controls, for males and females, respectively). Terminal body weights were approximately 10% lower in the animals at the highest dose than in the controls.

During the first half of the treatment period, mean food consumption was lower in males and females at the highest dose, relative to controls; this was considered to be treatment-related. Mean ratios of food consumption to body-weight gain were up to about 1.2-fold higher than control ratios in animals at the highest dose during the latter half of the study, indicating reduced food conversion efficiency. This was reflected in the lower body-weight gains in this group.

There was a dose-related increase in the mean absolute and relative spleen weights in males receiving cyprodinil at concentrations of 25–15 000 mg/kg in the diet, compared with controls. On histopathological examination, acute congestion of a moderate degree was noted in these animals. The study author cited insufficient exsanguinations as a probable cause of this finding, since there were no haematological or histopathological changes indicative of peripheral or central cardiovascular disorders. There was an increase in mean relative liver weights in males at 2500 and 15 000 mg/kg and in females at 250–15 000 mg/kg, although there was no dose–response relationship. There was also an increase in mean absolute and relative testes weights in all treated groups relative to the controls, (this was statistically significant for relative testicular weights in males at the highest dose), and absolute and relative thymus weights were significantly increased in all groups of treated females, although there was no dose–response relationship. In the absence of histopathological findings in any tissues, these observations were not considered to be toxicologically relevant.

One female at the lowest dose and three females at the highest dose had mottled lungs, which was reported to be of similar incidence as that observed in the colony. These changes correlated with foreign body granuloma in the animal at the lowest dose, and interstitial pneumonia in two animals at the highest dose. Therefore, these changes were not considered to be treatment-related.

Slight hepatocyte pigmentation, which was reported to be presumably of lipofuscin origin, was noted in three out of four males at the highest dose. Acute congestion of the spleen was noted in one male from each group at 250 and 2500 mg/kg, in two males at the highest dose, and in two and one females at 2500 and 15 000 mg/kg, respectively, which, as noted above, was considered to result from insufficient exsanguinations. No other findings were considered treatment-related. The NOAEL was considered to be 2500 mg/kg (66 mg/kg bw per day in males, 68 mg/kg bw per day in females) on the basis of decreased body-weight gains, decreased food consumption, and reduced food efficiency in males and females at

the LOAEL of 15 000 mg/kg (449.25 mg/kg bw per day in males, 446.37 mg/kg bw per day in females). The NOEL was 2500 mg/kg, equal to 66 mg/kg bw per day for males and 68 mg/kg bw per day for females (Altmann, 1992).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month study of carcinogenicity, groups of 70 male and 70 female Tif MAGf (SPF) albino mice were fed diets containing cyprodinil (purity, 99.6%) at a concentration of 0, 10, 150, 2000 or 5000 mg/kg (equal to 0, 1.15, 16.1, 212.4 or 630 mg/kg bw per day for males and 0, 1.08, 14.7, 196.3 or 558.1 mg/kg bw per day for females). Animals were examined at least once daily for signs of morbidity and mortality. Body-weight and food consumption were recorded weekly for the first 3 months, then monthly thereafter. Blood was collected from the orbital sinus of animals that had been fasted overnight (10 of each sex per dose per collection), under ether anaesthesia, at weeks 53 and 78 to assess haematology parameters. All animals that died during the study and those that were sacrificed moribund and at study termination were subjected to a detailed necropsy. A full histological examination was performed on all animals participating in the carcinogenicity part of the study, including those that died or were sacrificed before study termination. All organs from 10% of the animals of both sexes (selected at random), all target organs, all tumours, and all unusual lesions were re-evaluated by the reviewing pathologist. This study complied with GLP.

There was no effect on mortality, haematology parameters, and no clinical signs of toxicity. Survival was similar or greater in treated groups than in control groups, with males and females at the highest dose having the greatest survival rate of any of the groups (90–95%) at termination (Table 11). Mean body weights were significantly lower in males at the highest dose from week 8 until study termination, with an overall body-weight gain that was 10% lower than that in the control group. At the highest dose, there was a more progressive effect on body weight and body-weight gain in females than in males; body weights were significantly lower in this group compared with those in the control group from week 21 onward, with an overall body-weight gain that was 25% lower than that in the control group. This was considered to be treatment-related. Food consumption was difficult to assess in males, due to the high incidence of food spillage, particularly in the group receiving the highest dose. According to the study author, recalculating the group mean food consumption by excluding the values recorded when food wastage occurred resulted

Table 11. Number of survivors at termination of an 18-month study of carcinogenicity in mice fed diets containing cyprodinil

Dietary concentration (mg/kg)	Number of survivors	
	Male	Female
0 (control)	36/50	41/50
10	33/50	40/50
150	39/50	46/50
2000	39/50	43/50
5000	45/50*	48/50

From Fankhauser (1994a, 1994b)
*p = 0.028 in comparison with control group

in similar values for treated and control groups. Although there appeared to be no differences in food consumption, progressively higher food consumption ratios (g of food/kg bw per day) were noted in males and females at the highest dose throughout the study period, which indicated reduced food conversion efficiency in these groups, and was considered to be treatment-related.

There was a non-significant increase (8.7%) in the absolute mean liver weight in males at the highest dose and a significant increase (approximately 16%) in the mean liver to body weight ratios in males and females at the highest dose at termination, relative to controls. Relative mean kidney weight was also greater in females at the highest dose, although absolute kidney weights did not reveal any dose-response relationship.

There was a dose-related increase in the incidence of enlarged livers in males at 2000 and 5000 mg/kg. However, there was only a slight increase in the incidence of hepatic hypertrophy in the males at the highest dose relative to the controls, with no other liver histopathology. Furthermore, there was no clear treatment-related effect on liver necrosis in males, and the incidence of liver necrosis in females at the highest dose was, in fact, below that of the controls. No other macroscopic findings were treatment-related.

There was a dose-related increase in the incidence of minimal to marked focal and multifocal hyperplasia in pancreatic acinar cells in males at 2000 and 5000 mg/kg, which was statistically significant at 5000 mg/kg. In male mice, hyperplasia in pancreatic acinar cells was observed in 4/50, 4/50, 5/50, 8/50 and 14/50 animals at 0, 10, 150, 2000 and 5000 mg/kg, respectively. In female mice, hyperplasia in pancreatic acinar cells was observed in 3/50, 6/50, 2/50, 4/50 and 6/50 animals at 0, 10, 150, 2000 and 5000 mg/kg, respectively. In male mice, hyperplasia in pancreatic acinar cells was statistically significant only at the highest dose. It should be noted that incidences in all treated groups were higher than values for historical controls (range, 0–1.7% for both 18- and 24-month studies; data from 3 and 10 studies, respectively) provided in the study report. Additional data on historical controls provided by the sponsor show a range of 0–3.7% for incidence of hyperplasia in pancreatic acinar cells. Therefore, this was considered to be a treatment-related effect only in males at 5000 mg/kg. Incidences of pancreatic hyperplasia in all groups of females were also noted as being above those for historical controls (0% in 3 18-month studies, 0–3.9% in 10 24-month studies), but in the absence of a dose-response relationship, this was not considered to be treatment-related. The increased incidence of Harderian gland inflammation, observed in males only, was attributed to post-traumatic changes related to blood sampling, on the basis of morphological appearance and unilateral occurrence, and was considered to be an incidental finding. At 5000 mg/kg, females exhibited a significantly increased incidence of cystic kidneys and splenic haemosiderosis, and a non-significant increase in lymphohistiocytic infiltration of the kidney and splenic extramedullary haematopoiesis. The incidence and severity of haemosiderosis (Dunn, 1954; Hirouchi et al., 1994) and splenic extramedullary haematopoiesis generally increases with age, with a greater incidence of enhanced erythropoiesis in females than in males of certain mouse strains (Frith & Wiley, 1981). The study author suggested that there was a relationship between the increased incidence of haemosiderin pigment accumulation and the increased longevity of the animals at the highest dose, and further indicated that the lack of any indication of increased erythrocyte turnover supported this as an incidental finding, which was considered to be a valid conclusion. It was also indicated that the other changes noted were common occurrences in the colony, although data on historical controls were not provided to validate this statement.

There was no indication of carcinogenic potential at any dose. The NOAEL was 2000 mg/kg (212 and 196 mg/kg bw per day in males and females, respectively). The LOAEL was 5000 mg/kg (630 and 558 mg/kg bw per day in males and females, respectively) on the basis of the increase in the incidence of focal and multifocal hyperplasia of the exocrine pancreas in males, reduced body weights in males and females, increased relative kidney weights in females, and increased in relative liver weights in males and females. The NOEL was 2000 mg/kg (equal to 212 mg/kg bw per day for males and 196 mg/kg bw per day for females) (Fankhauser, 1994a, 1994b).

Rats

In a 24-month study of toxicity and carcinogenicity, groups of 50 male and 50 female Tif RAIf rats (plus groups of 20 males and 20 females that were used for laboratory investigations) were fed diets containing cyprodinil (active ingredient, 99.2–99.6%) at a concentration of 0, 5, 75, 1000 or 2000 mg/kg (equal to 0, 0.177, 2.7, 35.6 or 73.6 mg/kg bw per day for males, respectively, and 0, 0.204, 3.22, 41.2 or 87.1 mg/kg bw per day for females, respectively) for 24 months. Additional groups of 10 males and 10 females received the test diets for 12 months (interim sacrifice). Animals were examined at least once daily for signs of morbidity and mortality. Body weights and food consumption were recorded weekly for the first 3 months, then monthly thereafter. Water consumption was recorded monthly for the first 6 months; measurement was suspended thereafter, since no differences were found. Ophthalmological examinations were performed on all animals in the control groups and at the highest dose (the group being monitored for carcinogenicity) before the start of treatment and at 6, 12, 18, and 24 months. In addition, the eyes of all females were examined at termination. Haematological and clinical chemistry parameters were evaluated at weeks 13, 27, 53, 78, and 105. Urine analysis was performed at weeks 13, 27, 53, 78 and 105. All animals that died during the study and those that were sacrificed moribund and at study termination were subjected to gross necropsy. A full histological examination was performed on all animals in the group monitored for carcinogenicity and the group that was sacrificed in the interim only, including (when possible) those that died or were sacrificed before study termination. This study complied with GLP.

Survival was similar in treated and control groups, with approximately 50% of males and approximately 60% of females surviving in all groups, except in the group of males receiving the highest dose, which had the lowest survival rate of 46%. None of the clinical signs observed appeared to be treatment-related. Treated females in all groups showed an increase in frequency of cloudy or opaque eyes relative to controls, however, there was no dose–response relationship, no increase observed in males, and the observations were unilateral in 50% of affected females. Thus, this finding was considered to be incidental. There were no differences in body weight or body-weight gain between control and treated animals at any point during the 24-month study. There were no differences in food consumption or food conversion efficiency (g of food/kg bw per day) between control and treated groups over the entire study period. Urine analysis did not detect any treatment-related findings.

The most notable change in haematological parameters was a significant prolongation in prothrombin time in males at 2000 mg/kg at week 27 and in males at 1000 and 2000 mg/kg at week 53, although these values were within the upper physiological range provided in the company report. No differences were noted between control and treated groups at later time-points, and therefore the change in prothrombin time was of no toxicological concern. Any other changes in haematological parameters were sporadic, lacked

a dose-response relationship, and were noted at earlier time-points but not at termination, and therefore were not of concern.

At termination, plasma urea concentrations (4.67, 5.88, 5.60, 7.88, 8.70 nmol/l at 0, 5, 75, 1000, and 2000 mg/kg, respectively) were greater than the upper physiological limit (given as 6.94 nmol/l in the study report) in males at 1000 and 2000 mg/kg, although these values were not statistically significant, relative to controls. Cholesterol and phospholipid concentrations were significantly greater than controls in all groups of treated females at week 27 (with the exception of phospholipid concentrations in females at the lowest dose), although these were still within the physiological range. However, there were no significant or dose-related differences in these values at any other time over the 24-month study. Therefore, changes in these parameters were unlikely to be of toxicological significance. Any other changes in clinical chemistry parameters, in either males or females, were sporadic with no dose-response relationship, or were transient, and fell within the normal physiological range provided in the study report.

The only apparent treatment-related effect on organ weight was a significant increase (11%) in absolute and relative liver weights in males at the highest dose, compared with controls, at termination. Increased relative mean kidney weights males and females at the highest dose at the interim sacrifice and lower mean exsanguinated body weight in females at the highest dose were not observed in animals at termination. At the highest dose, absolute and relative kidney and adrenal weights were increased relative to controls in males, and the mean ovarian weight was increased in females at study termination; however, these differences were attributed to grossly enlarged organs in one or two animals for each tissue type, and were not considered to be treatment-related.

At gross necropsy, females at the highest dose exhibited an increased incidence of mottled lungs, which correlated with an accumulation of foam cells in lung alveoli, and an increase in ovarian cysts (all at terminal sacrifice), which was often noted in conjunction with ovarian atrophy (Table 12). These lesions are common occurrences in ageing animals and were not considered to be toxicologically significant. The incidence of ovarian atrophy in concurrent controls was 45%.

Selected non-neoplastic findings on microscopic histopathological examination are presented in Table 13. There was a dose-related increase in the incidence of spongiosis hepatitis (sinusoidal cystic dilation) in the liver of males at 1000 and 2000 mg/kg, which was considered to be toxicologically significant. This is a degenerative, multilobular, cystic liver change that affects primarily perisinusoidal cells of the liver. Excessive amounts of acid mucopolysaccharides and/or proteinaceous material are produced, leading to the development of large cavities characteristic of this lesion (Banasch et al., 1985, Eustis et al., 1990).

Table 12. Selected observations at gross necropsy of female rats given diets containing cyprodinil in a 24-month study of toxicity/carcinogenicity

Lesion	Dietary concentration (mg/kg)				
	0	5	75	1000	2000
Mottled lungs	6/80 (8.0%)	7/80 (9%)	8/80 (10%)	6/80 (8%)	16/79 (20%)
Cystic ovaries	2/80 (3%)	1/80 (1%)	2/80 (3%)	0/80 (0%)	9/80 (11%)

From Fankhauser (1994c, 1994d)

Table 13. Incidence of selected non-neoplastic lesions in a 24-month study of toxicity/carcinogenicity in rats given diets containing cyprodinil

Lesion	Sex	Dietary concentration (mg/kg)				
		0	5	75	1000	2000
Spongiosis hepatitis	Male	3/60 (5%)	3/60 (5%)	2/60 (3%)	10/60* ^{##} (17%)	14/60* ^{##} (23%)
Liver necrosis [interim sacrifice; main group]	Male	9/60 (15%) [2/10; 7/50]	9/60 (15%) [5/10; 4/50]	6/60 (10%) [1/10; 5/50]	9/60 (15%) [2/10; 7/50]	16/60 (27%) [5/10; 11/50 [#]]
Progressive nephropathy	Male	39/60 (65%)	40/60 (67%)	37/60 (62%)	39/60 (65%)	49/60* ^{##} (82%)
	Female	19/60 (32%)	22/60 (37%)	21/60 (35%)	16/60 (27%)	24/60 (40%)
Foam cells—lung	Female	18/60 (30%)	15/60 (25%)	16/60 (27%)	26/60 [#] (43%)	26/60 ^{##} (43%)

From Fankhauser (1994c, 1994d)

* $p < 0.05$; ** $p < 0.01$ (pairwise comparison); [#] $p < 0.05$, ^{##} $p < 0.01$ (positive trend)

Table 14. Selected neoplastic findings in rats given diets containing cyprodinil in a 24-month study of toxicity/carcinogenicity

Organ/tumour	Sex	Dietary concentration (mg/kg)					Historical controls
		0	5	75	1000	2000	
Liver	Male						
Adenomas		1/60	3/60	3/60	1/60	2/60	—
Carcinomas		0	0	1/60	1/60	2/60 [#]	—
Mammary gland	Female						
Fibroadenoma ^a		15/50	18/50	15/50	18/50	27/50* ^{##}	11–57% ^b

From Fankhauser (1994c, 1994d)

^aNumber of animals examined does not include interim sacrifice group

^bTen studies, conducted over 1984–1990

* $p < 0.05$, ** $p < 0.01$ (pairwise comparison); [#] $p < 0.05$, ^{##} $p < 0.01$ (trend)

An increased incidence of liver necrosis was noted in males at the highest dose, relative to the controls. However, this was attributed to a high incidence of recent necrosis, a peri-mortal artifact that was noted at the highest dose in 10 out of 11 males that were found dead. Therefore, this finding was not directly related to treatment. Progressive nephropathy was observed at a higher incidence in males at the highest dose, compared with the controls. However, the range for incidence of progressive nephropathy in historical controls of this strain of male rats was 25–88%, which excluded this lesion from being considered as a treatment-related finding. Although there was a higher incidence of lung alveolar foam cells in females at 1000 and 2000 mg/kg, relative to controls, this fell within the range of 5–54% for historical controls, and was not considered to be toxicologically significant.

Table 14 lists the incidence of selected neoplastic findings. Although the occurrence of hepatocellular carcinoma in males at the highest dose showed a significant positive trend ($p < 0.05$), the low incidence (2 out of 60 versus 0 out of 60 in controls) did not support this finding as a treatment-related effect (Table 17). There was an increased incidence of mammary gland fibroadenomas in females at the highest dose; the data indicated that the frequency fell within the range of 11–57% for historical controls (overall mean, 41%, from studies initiated in 1984–1990 and 40% for studies initiated in 1988–1990; in 5 of these 10 studies, the incidence of mammary gland fibroadenomas was $\geq 44\%$ or above). In studies initiated within 3 years of the present study (1988–1990), incidences of 44–57% were observed in 4 out of 10 studies. It should be noted that incidences in the concurrent control

group and in the three groups receiving cyprodinil at 5, 75 or 1000 mg/kg, were lower than those for historical controls in 9 out of 10 studies. In view of the data on historical controls, and the lack of any such findings in the study of carcinogenicity in mice, the mammary fibroadenomas were not considered to be treatment-related.

The NOAEL for toxicity was 75 mg/kg (2.7 mg/kg bw per day) on the basis of a dose-related increase in the incidence of spongiosis hepatitis in the liver of males at 1000 and 2000 mg/kg. There was no evidence of significant chronic toxic effects in females. The NOEL was 75 mg/kg, equal to 2.7 mg/kg bw per day for males and 3.22 mg/kg bw per day for females (Fankhauser, 1994c, 1994d).

2.4 Genotoxicity

Cyprodinil was tested in various assays for for genotoxicity in vivo and in vitro (Table 15). These studies complied with GLP. There was no evidence for gene mutations in assays for bacterial reverse mutation in vitro (in *S. typhimurium* and *E. coli*), or for gene mutation in Chinese hamster lung V79 cells or Chinese hamster ovary cells, either in the presence or absence of metabolic activation. Cyprodinil gave negative results in a test for mammalian cell DNA repair in vitro and in a test for micronucleus formation in mouse cells in vivo.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

A two-generation study of reproduction was carried out in Tif RAIf rats (one litter per generation). Groups of 30 males and 30 females received diets containing cyprodinil (purity, 99.5%) at a concentration of 0, 10, 100, 1000 or 4000 mg/kg (equal to 0, 0.7, 7.4, 74.4 or 294.7 mg/kg bw per day for males, respectively, and 0, 0.8, 8.2, 81.3 or 326.4 mg/kg bw per day for females, respectively; dietary concentrations in mg/kg bw per day are based on food consumption during the pre-mating period for F₀ animals) for two generations. Animals were fed test diets during the 10-week pre-mating period in both generations, then randomly allocated to mating pairs (1:1) for a maximum of 19 days. Upon detection of

Table 15. Results of studies of genotoxicity with cyprodinil

End-point	Test object	Concentration/dose	Purity (%)	Results	Reference
<i>In vitro</i>					
<i>S. typhimurium</i>					
Reverse mutation	TA98, TA100, TA1535 and TA1537; <i>E. coli</i> WP2 <i>uvrA</i>	20.0–5000 µg/plate in DMSO, ± S9	99.5	Negative	Ogorek (1990)
Gene mutation	V79 Chinese hamster lung fibroblast cells; <i>Hgp^{rt}</i> locus	6–150 µg/ml in DMSO, +S9 1.5–30 µg/ml in DMSO, –S9	99.5	Negative	Geleick (1990)
Chromosomal aberrations	Chinese hamster ovary cells	6.25–50 µg/ml in DMSO, +S9 3.13–25 µg/ml in DMSO, –S9	99.5	Negative	Strasser (1991)
DNA repair	Rat hepatocytes (Tif RAIf (SPF))	0.74–80 µg/ml in DMSO	99.5	Negative	Geleick (1991)
<i>In vivo</i>					
Micronucleus formation	Mouse bone-marrow cells	1250–5000 mg/kg bw, single dose by gavage	99.5	Negative	Ceresa (1990)

S9, 9000 × g supernatant fraction of Aroclor 1254-induced rat liver microsomes; DMSO, dimethylsulfoxide

vaginal sperm or a copulating plug, which was considered to be day 0 of gestation, females were moved to individual cages and males were returned to their original cages. Precautions were taken to avoid matings between siblings. F₁ litters were culled to four males and four females, if possible, on postnatal day 4. Animals were observed daily for clinical signs and mortality. Body weight and food consumption were recorded weekly. Necropsy was performed at termination. Selected organs were removed, weighed and histopathological examination was performed. This study complied with GLP.

No treatment-related clinical signs or mortalities were observed. Signs of toxicity at 4000 mg/kg consisted of a statistically significant decrease in body-weight gain (12.5%) in the F₀ females after the pre-mating period. Mean food consumption was decreased in F₀ females during the initial pre-mating phase. There were no treatment related effects on the reproductive parameters evaluated. The increased liver weight in males and females at 1000 (marginal) and 4000 mg/kg was not supported by histopathological findings; although these changes were treatment-related, they were not considered to be adverse. Mean absolute and relative kidney weights were significantly greater in F₀ males at 100–4000 mg/kg than those in controls, as were relative kidney weights in F₀ females at 1000 and 4000 mg/kg and in F₁ males at 4000 mg/kg. There were no histopathological findings in the kidney. However, subsequent histopathological examination revealed a marginal increase in the incidence and severity of renal tubular basophilia in F₀ males at the highest dose. Treatment with cyprodinil had no effect on proliferative activity in basophilic tubules and in unaffected cortical and medullary renal tubules according to assays for proliferating cell nuclear antigen (PCNA). Effects in kidneys were considered to be treatment-related; however, in the absence of significant histopathological findings (marginal renal tubule basophilia at the highest dose) or effect on proliferative activity, the increased organ weights were not considered to be toxicologically significant. At necropsy, liver enlargement was noted in males at the highest dose; other findings were considered to be incidental. The LOAEL for parental systemic toxicity was 4000 mg/kg (295 mg/kg bw per day) on the basis of decreased body-weight gain in the F₀ females during the pre-mating period. The NOAEL for parental systemic toxicity was 1000 mg/kg (74 mg/kg bw per day).

Signs of offspring toxicity included significantly lower weights for F₁ and F₂ pups at the highest dose during lactation; these continued to be lower than those of controls after weaning and after the pre-mating period (examined in F₁ generation only). The LOAEL for offspring toxicity was 4000 mg/kg (295 mg/kg bw per day) on the basis of decreased pup body weights (F₁ and F₂ generations) during lactation and continuing into adulthood for F₁ rats. The NOAEL for offspring toxicity was 1000 mg/kg (74 mg/kg bw per day). The NOEL was 100 mg/kg, equal to 7.4 mg/kg bw per day for males and 8.2 mg/kg bw per day for females (Khalil, 1993).

(b) Developmental toxicity

Rats

In a study of developmental toxicity, groups of 20–23 Tif RAIf (SPF) female albino rats were given cyprodinil (purity, 99.5%) at a dose of 0, 20, 200 or 1000 mg/kg bw per day in aqueous corn starch suspension (3%) by oral gavage on days 6–15 of gestation. Animals were observed daily for signs of morbidity, mortality, and abortion. Body weights were recorded daily. Food consumption was determined on days 0–6, 6–11, 11–16 and 16–21 of gestation and recorded as daily means. Females were sacrificed on day 21 of gestation and subjected to gross examination. Fetuses were delivered by caesarian section, weighed, sexed,

and examined for possible external, visceral and skeletal anomalies. This study complied with GLP.

One female in the group receiving the highest dose showed reduced locomotor activity on day 16 of gestation, which was considered to be treatment-related. No other clinical signs of toxicity were observed.

Significantly lower mean body weights were observed in females at the highest dose, beginning 1 day after initiation of treatment (day 7 of gestation) and continuing daily throughout the remainder of the study. Mean body-weight gains were significantly lower in females at the highest dose than in controls throughout the period of treatment.

Food consumption was significantly lower (58–68% of that in controls) in females at the highest dose throughout the entire dosing period; this correlated with the decrease in body-weight gain. Food consumption was comparable in control and females at the highest dose from days 16–21 of gestation. Food consumption was significantly lower (88% of that of the control group) in the group receiving a dose of 200 mg/kg bw during the first half of the period of treatment (days 6–11 of gestation); however, this did not appear to influence body weight in this group.

There were no other treatment-related observations. There were no differences in rates of pregnancy, mean numbers of corpora lutea, or implantation sites between groups. The LOAEL for maternal toxicity was 1000 mg/kg bw per day on the basis of lower body weight, body-weight gain and reduced food consumption. The NOAEL for maternal toxicity was 200 mg/kg bw per day.

There were non-significant increases in the number of early resorptions at the two higher doses compared with the controls (Table 16). The data on means for historical controls for early resorptions was reported to range from 2.9% to 10.5%. Although there appeared to be a dose–response relationship for early resorptions, the mean litter size was only slightly lower at the highest dose relative to the controls. Thus, a clear treatment-related effect could not be established.

One fetus (out of a total of 273 fetuses) in the group receiving the highest dose had omphalocele, which was not considered to be treatment-related. There were no visceral

Table 16. Summary of litter data in rats given cyprodinil by gavage

Parameter	Dose (mg/kg bw per day)			
	0	20	200	1000
No. of live litters	23	20	22	20
No. of live fetuses, male/female	168/164	129/153	167/142	141/132
No. of resorptions	13	13	20	23
Early resorptions (%)	10 (2.8)	13 (4.4)	20 (6.1)	22 (7.4)
Late resorptions (%)	3 (0.8)	0	0	1 (0.3)
Mean no. of resorptions per litter	0.6 ± 0.7	0.6 ± 0.9	0.9 ± 1.4	1.1 ± 1.3
Mean no. of pups per litter	14.4 ± 4.2	14.1 ± 2.6	14.0 ± 3.5	13.7 ± 2.8
Mean % of male pups	50.6	45.7	54.0	51.6
Mean fetal weight	5.3 ± 0.3	5.3 ± 0.3	5.5 ± 0.4	4.7 ± 0.5*

From Marty (1991a)
**p* < 0.01

abnormalities. There were significantly lower mean fetal weights (11%) at the highest dose compared with controls, as well as a significant increase in skeletal anomalies in this group relative to the controls, due to abnormal ossification (4 out of 20 litters in the group receiving the highest dose compared with 0 in any other group), primarily as absent or reduced ossification of metacarpal 5. These skeletal anomalies/variations were considered to represent a transient developmental delay that occurs secondary to the maternal toxicity observed at the highest dose. The LOAEL for developmental toxicity was 1000 mg/kg bw per day on the basis of lower mean fetal weights and an increased incidence of delayed ossification. The NOAEL for developmental toxicity was 200 mg/kg bw per day. There was no evidence of teratogenicity in this study at a dose of up to 1000 mg/kg bw per day, given during the period of major organogenesis. The NOEL was 200 mg/kg bw per day (Marty, 1991a).

Rabbits

In a study of developmental toxicity, 19 inseminated female rabbits (Russian Chbb:HM (SPF)) were given cyprodinil (purity, 99.5%) at a dose of 0, 5, 30, 150, or 400 mg/kg bw per day in aqueous corn starch suspension (3%) by gavage from days 7 to 19 of gestation. The animals were checked daily for mortality or clinical signs. Body-weight data were recorded daily. Food consumption was determined for days 0–4, 4–7, 7–12, 12–16, 16–20, 20–24, and 24–29 of gestation and recorded as daily means. Dams were sacrificed on day 29 of gestation. Macroscopic pathological examinations of the main organs were carried out at sacrifice. Fetuses were removed, weighed, sexed, and examined for external, visceral, and skeletal anomalies. This study complied with GLP.

All rabbits survived to termination on day 29 of gestation. No treatment-related clinical observations were reported. The mean maternal body weight for dams at 400 mg/kg bw per day was comparable to that for controls on day 7 of gestation, but decreased slightly (not statistically significant) after dosing began. Mean body-weight gain in dams at 400 mg/kg bw per day was –56 g between days 7 and 13 of gestation compared with –9 g in the controls (statistically significant, $p < 0.01$), and was 58% less than that in controls (controls, 40 g; high dose, 17 g) between days 13 and 19 (not statistically significant). The mean body-weight gain in this group for the entire treatment period (days 7–19) was significantly depressed ($p < 0.01$). On days 19–29 after treatment, the mean body-weight gain in the dams at 400 mg/kg bw per day was 35% greater than in controls (not significant). Mean gravid uterine weights were comparable across all groups. Food consumption was 34% less than that in controls (not statistically significant) on days 7–12 after insemination. This decrease paralleled the decreased body-weight gain for the first week of treatment. No treatment-related gross pathological findings were observed. No effects were noted on pregnancy parameters.

The LOAEL for maternal toxicity was 400 mg/kg bw per day on the basis of decreased body-weight gain. The NOAEL for maternal toxicity was 150 mg/kg bw per day.

At 400 mg/kg bw per day, fetuses exhibited indications of an increased incidence (not statistically significant) of a thirteenth rib. Although there were indications of developmental delays in fetal skeletons, such as poor or absent ossification of the sternebrae and metacarpals at all doses, only the development of the extra rib is likely to be compound-related. Uni- or bilateral hyperflexion of the carpal joint at 400 and 150 mg/kg bw per day (not statistically significant) was not associated with the skeletal changes. At the highest dose tested, a slight increase in the number of litters in which pups were born with an extra

(thirteenth) rib was observed in rabbits in the presence of maternal toxicity, an effect that was not considered to be toxicologically relevant. Therefore, the NOAEL for fetal development was 400 mg/kg bw per day, the highest dose tested. Under the study conditions used, cyprodinil was not teratogenic to rabbits at a dose of up to 400 mg/kg bw per day. The NOAEL for maternal toxicity was 150 mg/kg bw per day and the NOAEL for developmental toxicity was 400 mg/kg bw per day, the highest dose tested (Marty, 1991b).

2.6 *Special studies*

(a) *Effects on the kidney*

Increased absolute and relative kidney weights were observed in F₀ males given cyprodinil at a dietary concentration of 1000 and 4000 mg/kg in the two-generation study of reproduction in rats. In addition, a small increase in the incidence and severity of tubular basophilia was observed at 4000 mg/kg. In the study described below, formalin-fixed renal tissues from F₀ males in the two-generation study of reproduction were investigated by immunohistochemical/morphological analysis of PCNA. PCNA-stained sections were also used for determining proliferative activity in basophilic tubules. This study complied with GLP.

Basophilic tubules, mainly found in the cortex and the outer stripe of outer medulla, were characterized by one or more of the following features: cytoplasmic basophilia, pale and sometimes crowded nuclei, thickening of the basement membrane, infiltration with inflammatory cells (lymphocytes) and associated tubular dilatation with eosinophilic contents. Basophilic tubules as observed in this study therefore correspond to those spontaneous, age-related lesions in the laboratory rat that have mainly been interpreted as degenerative/regenerative tubules or early forms of progressive nephropathy. All other histopathological findings in the kidney were devoid of any treatment-related effect and there was no indication of the occurrence of putative pre-neoplastic kidney lesions in any group.

No effect of treatment was observed on the numerical density of tubular cell nuclei (number of tubular cell nuclei per mm²) in cortical and medullary compartments. In addition, the relationship between numerical density of tubular cell nuclei in the cortex and in the medulla was unchanged.

Treatment with cyprodinil did not influence the number of tubular cell nuclei that stained positive for PCNA in unaffected cortex or medulla (outer stripe of outer medulla). In addition, no difference was noted in the numerical density of PCNA-positive nuclei in the cortex and in the medulla between treated and control animals. In all animals, the number of PCNA-positive nuclei was significantly higher in cortical than in medullary renal tubules.

Sections from those animals with basophilic tubules (identified by staining with haematoxylin and eosin) were stained for PCNA and morphometrically examined to determine the PCNA-labelling index in basophilic and in adjacent unchanged tubules. Generally, the labelling index was more than 10-fold higher in basophilic tubules than in adjacent unchanged tubules. No treatment-related effect on the labelling index of basophilic and adjacent unchanged tubules was noted. Additionally, the relationship between the PCNA-labelling index in basophilic and in adjacent unchanged tubules remained uninflu-

enced by treatment with cyprodinil. Therefore, cyprodinil at a dietary concentration of up to 4000 mg/kg did not increase proliferative activity (as measured by PCNA-labelling index) in basophilic and adjacent unchanged tubules.

Under the study conditions used, treatment with cyprodinil resulted in a slight increase in the severity of tubular basophilia in F₀ males at the highest dose. These basophilic tubules mainly correspond to degenerative/regenerative or chronic progressive nephropathic lesions, in terms of histopathology and proliferative activity. Treatment with cyprodinil had no effect on proliferative activity in basophilic tubules or in unaffected cortical and medullary renal tubules (Weber, 1997).

(b) Neurotoxicity

Rats

In a range-finding study of acute oral neurotoxicity, groups of three male and female Tif RAIf (SPF) rats were given a single dose of cyprodinil (purity, 99.2%) in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) at 0, 300, 1000 or 2000 mg/kg bw, administered by gavage in a volume of 10 ml/kg bw. Animals were examined daily for clinical signs and mortality. Body weights were recorded weekly and food consumption was recorded twice per week. All animals were subjected to a functional observational battery (FOB), and motor activity evaluations were conducted before the treatment and on days 1 (1, 2, 4, 6, and 8 h after administration), 2, 5, and 8. Necropsies were not performed. This study complied with GLP.

Mortality, body weight, and body-weight gains were unaffected by the test substance. Food consumption was transiently reduced in animals at the highest dose (2000 mg/kg bw). At this dose, reduced activity, decreased muscle tone, lowered responsivity, hunched posture/gait, and piloerection and in some animals, palpebral closure and slight gait abnormalities, were observed in FOB parameters. These effects appeared first at 1 h after dosing, peaked at about 2 h after dosing, and disappeared on day 2. The same clinical findings were recorded for one female at 1000 mg/kg bw. The gait anomalies noted in two females at the highest dose were attributed to non-specific systemic toxicity as they were observed soon after dosing and were not present later, at the time of peak effects. The signs and symptoms observed at the limit dose in this study were attributed to the systemic toxicity of cyprodinil and were not considered to be an indication of any specific neurotoxic effects of cyprodinil. The LOAEL was 1000 mg/kg bw on the basis of systemic toxicity observed in one female. The NOAEL was 300 mg/kg bw and the NOEL was 300 mg/kg bw (Classen, 1996).

In a study of acute neurotoxicity, groups of 10 male and 10 female Tif RAIf (SPF) rats were given a single dose of cyprodinil (purity, 99.2%) in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) at 0, 200, 600, or 2000 mg/kg bw, administered by gavage in a volume of 10 ml/kg bw. Animals were examined daily for clinical signs and mortality. Body weights were recorded weekly and food consumption twice per week. All animals were subjected to a FOB and motor activity evaluations were conducted before treatment and on days 1 (2 h after dosing), 8, and 15. Upon study termination, neuropathological examination of perfusion-fixed tissues from the central and peripheral nervous system was conducted on five rats of each sex from the control group and the group receiving the highest dose. Data on positive controls were not provided. This study complied with GLP.

One male and one female at 600mg/kgbw died on day 2 and day 18, respectively. No other mortalities were observed. The presence of clear fluid in the thoracic cavity of the dead male rat is indicative of intubation error. Hunched posture was seen in all females at 600 and 2000mg/kgbw, which was accompanied by piloerection and reduced activity in most animals. In addition, hunched posture was seen in five females at 200mg/kgbw from day 1 to day 3, which is not considered as an adverse finding since no clinical signs were noted in two other studies of neurotoxicity (Classen, 1996, 1998) at comparable doses. All signs disappeared in 3 to 4 days. Body weight, body-weight gain and food consumption were comparable in animals in treated groups and controls.

At the time of peak effects, reduced activity, hunched posture, piloerection, and increased responsiveness to sensory stimuli were observed in females at 600 and 2000mg/kgbw and persisted until day 4. A dose-related decrease in body temperature was noted in all treated groups. The decrease in body temperature was statistically significant for all treated males and for females at 600 and 2000mg/kgbw. The decrease in body temperature at 200mg/kgbw was not considered to be biologically relevant since it was minimal, and not seen in the other study of neurotoxicity at a similar dose (Classen, 1998). No treatment-related neuropathic changes were observed. The signs and symptoms observed at 600 and 2000mg/kgbw (the limit dose) in this study were attributed to the systemic toxicity of cyprodinil and were not indicative of neurotoxic effects of cyprodinil.

The LOAEL was 600mg/kgbw on the basis of reduced activity, hunched posture, piloerection, increased responsiveness to sensory stimuli, and hypothermia. The NOAEL was 200mg/kgbw and the NOEL was 200mg/kgbw (Classen, 1997a).

A study of acute neurotoxicity in rats was conducted to verify the occurrence of minimal hypothermia and hunched posture seen at 200mg/kgbw in previous studies and to identify a clear NOEL for these findings. In this study, groups of 10 male and 10 female Tif RAIf (SPF) rats were given a single dose of cyprodinil (purity, 99.2%) in carboxymethylcellulose (0.5%w/v) and aqueous polysorbate 80 (0.1%w/v) at 0, 20, 60, or 200mg/kgbw, administered by gavage in a volume of 10ml/kgbw. Animals were examined daily for clinical signs and mortality. Body weights were recorded weekly and food consumption was recorded twice per week. All animals were subjected to a FOB and motor activity evaluations were conducted before treatment and on days 1 (2h after dosing) and 8. The study was terminated on day 10 because there were no treatment-related effects. Gross necropsy and histopathology were not performed. Data on positive controls were not provided. This study complied with GLP.

Mortality, clinical signs, body weights, body-weight gains, food consumption, FOB parameters, and motor activity were unaffected by the test substance. A statistically significant reduction in body temperature was observed on day 8 in males at the highest dose (-0.2°C). This effect was considered to be incidental and toxicologically irrelevant because it was minimal and was not seen at the time of peak effects. No evidence of neurotoxicity was observed at any dose tested. The NOEL was 200mg/kgbw, the highest dose tested (Classen, 1998).

In a study of short-term neurotoxicity/toxicity, groups of 10 male and 10 female Tif RAIf (SPF) rats were given diets containing cyprodinil (purity, 99.2%) at a concentration of 0, 80, 800, or 8000mg/kg (equivalent to 0, 5.8, 54.5, and 601mg/kgbw per day, for males, respectively, and 0, 6.3, 58.7 and 631mg/kgbw per day for females, respectively) for 13

weeks. Animals were observed daily for clinical signs and mortality. Body weight and food consumption were recorded weekly. All animals were evaluated for FOB and motor activity at weeks -1, 4, 8, and 13. Upon study termination, neuropathological examination of perfusion-fixed central and peripheral nervous tissues was conducted on five rats of each sex from the control group and from the group receiving cyprodinil at 8000mg/kg. Pituitary glands were also examined in five rats of each sex from the control group and from the group receiving cyprodinil at 8000mg/kg. Data on positive controls were not provided. This study complied with GLP.

There were no deaths. No toxicologically relevant clinical signs or changes in behaviour were observed during the study. The body-weight gain of the males and females at the highest dose was reduced compared with that of the controls. A transient decrease in food consumption was observed in the animals at the highest dose in the first week of treatment. Observational and functional tests indicated no toxicologically relevant deviation from the controls. At week 13, mean landing foot splay was increased in male rats at 800mg/kg dose, however, this finding was considered to be incidental in view of the absence of any other effects and the fact that there was no dose-response relationship. No treatment-related effects on the various motor activity parameters were detected. The absolute and relative liver weights were increased in animals of both sexes at the highest dose and absolute and relative kidney weights were increased in females at the highest dose.

At necropsy, there were no macroscopic findings that indicated adverse treatment-related effects. An increased incidence of hepatocellular hypertrophy was observed in males and females at the highest dose. An increased incidence of chronic tubular lesions, classified as slight with respect to severity, was observed at the highest dose. In addition, an increased incidence of tubular casts of a slight degree of severity were observed at the highest dose. There was also an increased incidence of slight hypertrophy of follicular epithelial cells in the thyroid gland in animals at the highest dose. All five males examined in the control group and the group receiving the highest dose showed hypertrophy of the pars distalis, of moderate to marked severity. In females, two out of five rats at the highest dose showed thyroid hypertrophy, classified as being of minimal severity. All hypertrophic cells were positive for thyroid-stimulating hormone (TSH), but in all animals examined, additional TSH-positive cells existed that were not hypertrophic. No relevant differences in the number of TSH-positive cells in control and treated groups were found, while males generally had more TSH-positive and hypertrophic cells than females. Histopathological examination did not reveal any additional treatment-related abnormalities.

No evidence for neurotoxicity was observed at any dose tested. The LOAEL for this study was 8000mg/kg (equal to 601 and 631mg/kgbw per day for males and females, respectively) on the basis of liver, kidney and thyroid histopathology, reduced body-weight gain and food consumption, and increased absolute and relative liver and kidney weights (females only). The NOAEL for this study was 800mg/kg (equal to 54.5 and 58.7mg/kg bw per day for males and females, respectively). The NOEL for neurotoxicity was 8000mg/kg, (equal to 601mg/kgbw per day for males and 631mg/kgbw per day for females) (Classen, 1997b).

(c) *Studies on metabolites*

(i) *Acute toxicity*

CGA 249287 (4-cyclopropyl-6-methyl-pyrimidine-2-ylamine)

CGA 249287 is formed as a result of the cleavage of the phenyl group from the active substance, cyprodinil. It was identified in rats and also detected in studies of photolysis in soil and water, in plants such as tomato, wheat, apple, potato, peaches, and in goats and hens. In rat liver, approximately 0.014% of the administered dose of cyprodinil was recovered as CGA 249287.

In a study of acute oral toxicity, five male and five female young adult Tif RAIf (SPF) rats that had been fasted overnight were given CGA 249287 technical (purity, 99.8%), at a dose of 2000 mg/kg bw by oral administration. The test material was suspended in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) and administered in a volume of 10 ml/kg bw. Animals were observed daily for clinical signs and mortality. Body weights were recorded at the start of the study and weekly thereafter. Treated animals were observed for 14 days. This study complied with GLP.

One male was found dead 1 day after dosing. There were no other mortalities. Most animals showed the following signs: piloerection, hunched posture, dyspnoea, reduced locomotor activity and chromodacryorrhoea, with some of these symptoms appearing as soon as 1 h after dosing. Symptoms were no longer evident by day 6 (females) or day 7 (males). All survivors gained weight during days 0–7, and again during days 7–14.

The only finding at gross necropsy performed on the rat that died was a spotted thymus. No abnormalities were found in the other animals at necropsy.

The oral LD₅₀ for male and female rats was >2000 mg/kg bw (Hartmann, 1992).

CGA 275535 3-(4-cyclopropyl-6-methyl-pyrimidine-2-ylamino)-phenol

CGA 275535 is formed as a result of hydroxylation at position 3 of the phenyl ring of cyprodinil. It was found in studies of soil degradation, and in plants such as wheat and potato (amounts not considered toxicologically relevant).

In a study of acute oral toxicity, five male and five female young adult Hanlbm:WIST (SPF) rats that had been fasted overnight were given CGA 275535 technical (metabolite of cyprodinil; purity, 99.0%), at a dose of 2000 mg/kg bw by oral administration. The test material was suspended in carboxymethylcellulose (0.5% w/v) and aqueous polysorbate 80 (0.1% w/v) and administered in a volume of 10 ml/kg bw. Animals were observed daily for clinical signs and mortality. Body weights were recorded at the start of the study and weekly thereafter. Treated animals were observed for 14 days. This study complied with GLP.

There were no clinical signs of toxicity or mortality. No effects on body weights or body-weight gains were observed. Gross necropsy did not reveal any abnormalities. The acute oral LD₅₀ of CGA 275535 was >2000 mg/kg bw (Sommer, 2000a).

NOA 422054 (4-cyclopropyl-6-hydroxymethyl-pyrimidine-2-ylamine)

NOA 422054 is formed as a result of hydroxylation at the pyrimidine-methyl group and cleavage of the phenyl ring of cyprodinil. NOA 422054 was detected in studies of crop

rotation and is considered to be a hydroxy-methyl derivative of the plant, soil and rat metabolite, CGA 249287.

In a study of acute oral toxicity, five male and five female young adult Hanlbm:WIST (SPF) rats that had been fasted overnight were given NOA 422054 technical (metabolite of cyprodinil; purity, 99.0%) at a dose of 2000 mg/kg bw by oral administration. The test material was suspended in carboxymethylcellulose (0.5% w/v) in aqueous polysorbate 80 (0.1% w/v) and administered in a volume of 10 ml/kg bw. Animals were observed daily for clinical signs and mortality. Body weights were recorded at the start of the study and weekly thereafter. Treated animals were observed for 14 days. This study complied with GLP.

One female died on day 1 after treatment with a dose of 2000 mg/kg bw. There were no other mortalities. At 2000 mg/kg bw, hunched posture and ventral recumbency were observed in both male and female rats. Increased dyspnoea, and hypoactivity were also observed in male and female rats in this group. One female was seen pushing her head through bedding. All surviving animals appeared to be normal by day 2. No effects on body weights or body-weight gains were observed. Gross necropsy did not reveal any abnormalities, except fluid in the small intestine and the stomach in one female. The acute oral LD₅₀ of NOA 422054 was >2000 mg/kg bw (Sommer, 2000b).

CGA 321915 4-cyclopropyl-6-methyl-pyrimidin-2-ol

CGA 321915 is formed as a result of hydroxylation and cleavage of the aminophenyl moiety of cyprodinil. It was found in studies of soil degradation and photolysis and in plants such as wheat.

In a study of acute oral toxicity, five male and five female young adult Tif RAIf (SPF) rats that had been fasted overnight were given CGA 321915 technical (metabolite of cyprodinil; purity, 94.0%), at 2000 mg/kg bw by oral administration. The test material was suspended in distilled water and administered in a volume of 10 ml/kg bw. Animals were observed daily for clinical signs and mortality. Body weights were recorded at the start of the study and weekly thereafter. Treated animals were observed for 14 days. This study complied with GLP.

There were no mortalities. At 2000 mg/kg bw, piloerection and hunched posture was reported in all animals; this subsided within 1 day. No effects on body weights or body-weight gains were observed. Gross necropsy did not reveal any abnormalities. The acute oral LD₅₀ of CGA 321915 was >2000 mg/kg bw (Winkler, 1996b).

(ii) Short-term studies of toxicity

CGA 249287 (4-cyclopropyl-6-methyl-pyrimidine-2-ylamine)

In a 90-day study of toxicity, groups of 12 male and 12 female Alpk:ApfSD (Wistar-derived) rats were given diets containing CGA 249287 (metabolite of cyprodinil, purity, 100%) at a concentration of 0, 300, 1000, or 4000 mg/kg (equal to 0, 23.9, 79.5, and 304.8 mg/kg bw per day for males and 0, 27.2, 90.5, and 342.6 mg/kg bw per day for females) for 90 days. Animals were observed daily for clinical signs and mortality. Body weights and food consumptions were recorded weekly. All animals were evaluated for FOB and motor activity during week 13. Ophthalmoscopic examinations were performed on all animals before treatment and on controls and animals in the group receiving the highest dose before sacrifice. Haematological, clinical chemistry and urine analysis were performed at termi-

nation. All treated animals were sacrificed, selected organs were removed and weighed, and histopathological examination was performed. This study complied with GLP.

No mortalities were observed. No treatment-related effects on clinical signs of toxicity, ophthalmoscopic examination, haematology parameters, clinical chemistry, clinical pathology or urine analysis were observed. There were no treatment-related effects on FOB, landing foot splay measurements, grip strength, time to tail flick or motor activity. At the highest dose, a slight increase in the value for forelimb grip was noted in males, which was considered to be incidental and unrelated to treatment because no effects were seen in females, no effects were seen on hindlimb splay in either sex, and the values for males were within the range for historical controls.

At 4000 mg/kg, body weights were reduced by 16% for males and 7% for females, compared with those for concurrent controls. Similarly, body-weight gains for male and female rats at 4000 mg/kg were reduced by 26% for males and 17% for females, compared with those for controls. Food consumption of animals was also reduced by 15–17% at 4000 mg/kg, compared with controls. There were no effects on body weight, body-weight gain or food consumption in other treated groups. There was a reduction in the severity of intratubular microlithiasis in the kidneys of females at 4000 mg/kg, which was considered to be caused by reduced food consumption and not directly related to treatment.

The LOAEL was 4000 mg/kg (304.8 and 342.6 mg/kgbw per day for males and females, respectively) on the basis of reduced body weight, body-weight gain and food consumption. The NOAEL was 1000 mg/kg (79.5 and 90.5 mg/kgbw per day for males and females, respectively). The NOEL was 1000 mg/kg, equal to 79.5 mg/kgbw per day for males and 90.5 mg/kgbw per day for females (Milburn, 2001).

(iii) Genotoxicity

CGA 249287 (4-cyclopropyl-6-methyl-pyrimidine-2-ylamine)

In assays for reverse gene mutation in bacteria, cultures of four *Salmonella typhimurium* histidine-deficient (*his*⁻) strains (TA1535, TA1537, TA98 and TA100) and one tryptophan-deficient (*try*⁻) strain of *Escherichia coli* (WP2 *uvrA*) were exposed to CGA 249287 (active ingredient, 99.5%; in dimethylsulfoxide, DMSO) for 48 h at 37 ± 1.5°C to CGA 249287 at five concentrations ranging from 312.5 to 5000 µg/plate, in the presence and absence of metabolic activation from S9 (9000 × g supernatant of Aroclor 1254-induced rat liver microsomes). While some cultures were exposed to the vehicle (DMSO) alone, other cultures were treated with known mutagens and served as positive controls. At harvest, the frequency of reversion to prototrophy (*his*⁺, *try*⁺) in test cultures was compared to that in vehicle controls. This study complied with GLP.

Slight cytotoxicity was observed in *E. coli* WP2 *uvrA* at the highest concentration in the absence of metabolic stimulation, but at no concentration of test substance in either assay was the frequency of revertant (*his*⁺ and *try*⁺) colonies greater than that for the negative control (DMSO) in either the presence or the absence of metabolic activation. All positive controls reacted with marked increases in the frequency of revertants.

Thus, CGA 249287 (a metabolite of cyprodinil) was negative for induction of reverse mutation in these bacterial cultures, under the given experimental conditions (Hertner, 1992).

In two independent assays for mammalian cell gene mutation in vitro, L5178Y *Tk*^{+/-} mouse lymphoma cells were exposed to CGA 249287 (purity, 100%) in DMSO at a dose of up to 1492 µg/ml, with and without exogenous metabolic activation from S9 (the 9000 × g supernatant of Aroclor-induced liver microsomes from male Sprague Dawley rats). This study complied with GLP.

No reproducible increases in frequency of mutation at the *Tk* locus were observed in cultures treated with CGA 249287 technical, with or without metabolic activation. The positive controls, ethyl methanesulphonate and benzo[a]pyrene, induced a marked and significant increase in the frequency of mutation in both assays (Clay, 2001).

In an assay for chromosomal aberrations, Chinese hamster ovary CHO-CCL 61 cells were exposed to CGA 249287 technical (metabolite of cyprodinil; purity, 100%) in DMSO in two independent assays. In the initial assay, cells were exposed to CGA 249287 at a concentration of 0, 350, 700 or 1400 µg/ml for 3 h, with or without metabolic activation, followed by a further incubation of 18 h. In the confirmatory assay, cells were exposed to CGA 249287 at a concentration of 0, 175, 350 or 700 µg/ml for 21 h in the absence of metabolic activation and harvested immediately after exposure; or cells were exposed to CGA 249287 at a concentration of 0, 350, 700 or 1400 µg/ml for 3 h in the presence of metabolic activation, followed by a recovery period of 18 h. Metabolic activation was provided by the S9 fraction from Aroclor 1254-induced male HanIbm:WIST (SPF) rat livers. This study complied with GLP.

CGA 249287 technical was tested at concentrations up to those that were cytotoxic. In the initial assay, a statistically significant increase in the number of metaphases with aberrations was observed at 1400 µg/ml in the absence of metabolic activation. This increase in aberrations was within the range for historical controls and did not fulfil the criteria for a positive response; it is therefore not considered to be treatment-related. No statistically significant increase in the number of metaphases with specific aberrations was observed in the confirmatory assay in the absence of metabolic activation. No statistically significant increase in the number of metaphases with specific aberrations was observed in either the initial or the confirmatory assay, in the presence of metabolic activation. The positive controls resulted in a clearly increased number of aberrant metaphases in all experiments. There was no evidence that the test material increased the incidence of chromosome aberrations compared with controls (Ogorek, 2001).

CGA 275535 3-(4-cyclopropyl-6-methyl-pyrimidine-2-ylamino)-phenol

In repeat (initial and confirmatory) assays for reverse gene mutation in bacteria, cultures of five histidine-deficient (*his*⁻) strains of *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) and the tryptophan-deficient (*try*⁻) strain (WP2 *uvrA*) of *E. coli* were exposed to CGA 275535 technical (metabolite of cyprodinil; purity, 99%, in DMSO) for 48 h at 37 ± 1.5°C at concentrations in the range of 62.5–5000 µg/plate in the presence of metabolic activation (provided by S9, the 9000 × g supernatant of Aroclor 1254-induced rat liver), and in the range of 15.6–1000 µg/plate in the absence of metabolic activation by both the standard plate assay (initial trial, ±S9; confirmatory experiment, -S9), and with preincubation (confirmatory experiment, +S9) were used. Cultures exposed to the solvent alone (DMSO) served as negative controls, while cultures treated with known mutagens served as positive controls. At harvest, numbers of revertant colonies (*his*⁺, *try*⁺) were compared with those for controls treated with DMSO. This study complied with GLP.

Reproducible inhibition of growth occurred with or without metabolic activation in WP2 *uvrA* cultures treated with CGA 275535 at the highest concentration (2000 µg/plate), as shown by a reduction in the number of revertant (*try*⁺) colonies. In neither experiment was there any increase in revertant (*his*⁺ or *try*⁺) colonies at any concentration, in either the presence or absence of metabolic activation. In contrast, all positive controls exhibited marked increases in numbers of revertants.

Therefore, neither CGA 275535 technical nor its metabolites induced gene mutations in the bacterial strains assayed (Deparade, 2001).

NOA 422054 (4-cyclopropyl-6-hydroxymethyl-pyrimidine-2-ylamine)

In repeat (initial and confirmatory) assays for reverse gene mutation in bacteria, cultures of five histidine-deficient (*his*⁻) strains of *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) and the tryptophan-deficient (*try*⁻) strain (WP2 *uvrA*) of *E. coli* were exposed to NOA 422054 technical (metabolite of cyprodinil; purity, 99.0%; in DMSO) for 48 h at 37 ± 1.5°C at five concentrations ranging from 312.5 to 5000 µg/plate, in the presence and absence of metabolic activation from S9 (the 9000 × g supernatant of Aroclor 1254-induced rat liver) in both the standard plate assay (initial trial, ±S9; confirmatory experiment, -S9), and with preincubation (confirmatory experiment, +S9). Cultures exposed to the solvent alone (DMSO) served as negative controls, while cultures treated with known mutagens served as positive controls. At harvest, the numbers of revertant colonies (*his*⁺, *try*⁺) were compared with those for controls treated with DMSO. This study complied with GLP.

Reproducible growth inhibition occurred both in the presence and absence of metabolic activation in WP2 *uvrA* cultures at the highest concentration, 5000 µg/plate), as shown by a reduction in the number of revertant (*try*⁺) colonies. No precipitation was found in any test culture at any concentration. In neither experiment was there any increase in revertant (*his*⁺ or *try*⁺) colonies at any concentration, in either the presence or absence of metabolic activation. In contrast, all positive control cultures exhibited marked increases in number of revertants.

Therefore, neither NOA 422054 technical nor its metabolites induced gene mutations in the bacterial strains assayed (Deparade, 2000).

CGA 321915 (4-cyclopropyl-6-methyl-pyrimidin-2-ol)

In repeat (initial and confirmatory) assays for reverse gene mutation in bacteria, cultures of five histidine-deficient (*his*⁻) strains of *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) and the tryptophan-deficient (*try*⁻) strain (WP2 *uvrA*) of *E. coli* were exposed to CGA 321915 technical (metabolite of cyprodinil; purity, 94.0%; in distilled water) for 48 h at 37 ± 1.5°C at five concentrations ranging from 312.5 to 5000 µg/plate in the presence and absence of metabolic activation from S9 (9000 × g supernatant of Aroclor 1254-induced rat liver) by both the standard plate assay (initial trial, ±S9; confirmatory experiment, -S9), and with preincubation (confirmatory experiment, +S9). This study complied with GLP.

Reproducible growth inhibition occurred in both activated and nonactivated WP2 *uvrA* cultures treated at the highest concentration (5000 µg/plate), as shown by a reduction in the number of revertant (*try*⁺) colonies. No precipitation was found in any test culture at any

concentration. In neither experiment was there any increase in revertant (*his*⁺ or *try*⁺) colonies at any concentration, in either the presence or absence of metabolic activation. In contrast, all positive controls exhibited marked increases in number of revertants.

Therefore, neither CGA 321915 technical nor its metabolites induced gene mutations in the bacterial strains assayed (Ogorek, 1996).

3. Observations in humans

Available information on medical surveillance of workers employed in a pilot plant indicated that three employees (the total number of employees potentially exposed was not reported) in the laboratory area showed symptoms of flush, sensation of warmth and swelling of eyelids when weighing this substance. It was considered that these symptoms were caused by exposure to cyprodinil, which has the potential to cause slight eye irritation and skin sensitization, as reported in studies in animals. In a plant carrying out large-scale production of cyprodinil, one case of accidental exposure to the eye was reported. The affected person suffered slight conjunctivitis. After medical treatment, the exposed worker returned to work (Lorez & Schulze-Rosario, 2001).

In a published study using the alkaline comet assay, DNA damage was determined for four groups of farmers before and after one day of spraying of pesticides. The farmers were handling chlorothalonil, isoproturon, and triazoles in this study and exposure to cyprodinil occurred only during mixing. There was no evidence for DNA damage in the group of farmers exposed to cyprodinil and other pesticides.

No epidemiological studies were available in the published literature (Lebailly et al., 1998).

Comments

In studies of metabolism in rats, radiolabelled cyprodinil administered by gavage as a single dose of 0.5 or 100 mg/kg bw, or as repeated doses of 0.5 mg/kg bw per day for 14 days, was rapidly absorbed from the gastrointestinal tract and excreted. Approximately 75% (range, 71–85%) of an orally administered dose was absorbed over 48 h. At a dose of 0.5 and 100 mg/kg bw, two plasma level maxima of radioactivity were observed at approximately 0.5–1 h and 8–12 h, probably caused by reabsorption of material excreted in the bile. Approximately 92–97% of the administered dose was eliminated within 48 h in the urine (48–68%), faeces (29–47%), and bile (accounting for up to 35.4% of the dose in cannulated rats), with elimination being almost complete by day 7. Seven days after single or repeated oral administration at the lower dose, total tissue residues accounted for 0.15–0.60% of the administered dose. Cyprodinil was primarily metabolized by hydroxylation of the phenyl and pyrimidine rings and methyl group, and excreted mainly as glucuronide or sulfate conjugates in urine, faeces and bile. Approximately 3–8% of the parent compound was detected in the faeces. Excretion, distribution and metabolite profiles were essentially independent of dose, pretreatment and site of radiolabel, although there were some quantitative sex-dependent differences in urinary metabolites.

Cyprodinil has low toxicity when administered by the oral, dermal or inhalation routes. LD₅₀ values after oral administration were >2000 and >5000 mg/kg bw in rats and mice, respectively. The LD₅₀ in rats treated dermally was >2000 mg/kg bw. The LC₅₀ in rats

treated by inhalation was $>1.20\text{ mg/l}$ (the highest attainable concentration) after an exposure of 4 h. Clinical signs of toxicity such as piloerection, hunched posture, dyspnoea, and reduced locomotor activity were seen. Cyprodinil was not a dermal or ocular irritant, but was a skin sensitizer.

In short-term studies in mice, rats and dogs, administration of cyprodinil in the diet or by gavage resulted in reduced body-weight gain and reduced food consumption at a dose of 6000 mg/kg (equal to $849\text{ mg/kg bw per day}$), $1000\text{ mg/kg bw per day}$, and $\geq 15\,000\text{ mg/kg}$ (equal to $446\text{ mg/kg bw per day}$) in mice, rats and dogs, respectively. The major target organs were the liver, kidney, and thyroid in rats, and the liver in mice and dogs. Increases in liver weights were observed in mice at 6000 mg/kg (equal to $849\text{ mg/kg bw per day}$) and in rats at $\geq 2000\text{ mg/kg}$ (equal to $134\text{ mg/kg bw per day}$). Increases in thyroid weight were observed in rats at $\geq 2000\text{ mg/kg}$ (equal to $134\text{ mg/kg bw per day}$). Some mild to moderate histopathological changes in the liver, such as hepatocyte hypertrophy and multifocal single cell hepatocyte necrosis, were seen in mice and rats at $\geq 2000\text{ mg/kg}$ (equal to $25\text{ mg/kg bw per day}$ in mice and equal to $134\text{ mg/kg bw per day}$ in rats). In the kidneys, adverse effects were manifested in rats as chronic tubular lesions and chronic kidney inflammation at $\geq 2000\text{ mg/kg}$ (equal to $134\text{ mg/kg bw per day}$). The NOAEL in a 90-day study of toxicity in mice was 500 mg/kg (equal to $73.3\text{ mg/kg bw per day}$). The NOAEL in a 28-day study in rats treated by gavage was $100\text{ mg/kg bw per day}$. The NOAEL in a 90-day study of toxicity in rats was 300 mg/kg (equal to $19\text{ mg/kg bw per day}$). The NOAELs in a 90-day and a 1-year study of toxicity in dogs were 7000 mg/kg (equal to $210\text{ mg/kg bw per day}$) and 2500 mg/kg (equal to $66\text{ mg/kg bw per day}$), respectively.

In long-term studies of toxicity and carcinogenicity in mice and rats, there were no treatment-related neoplastic findings. In mice, a slightly increased incidence of hyperplasia in acinar cells of the exocrine pancreas in males was observed at the highest dose tested, 5000 mg/kg (equal to $558\text{ mg/kg bw per day}$). The NOAEL for systemic toxicity in mice was 2000 mg/kg (equal to $196\text{ mg/kg bw per day}$), on the basis of an increase in the incidence of focal and multifocal hyperplasia of the exocrine pancreas in males, reduced body weights in males and females, increased relative kidney weights in females, and increased relative liver weights in males and females, seen at the highest dose tested. In rats, histopathological changes in the liver (spongiosis hepatitis) and increased liver weights were observed at $\geq 1000\text{ mg/kg}$ (equal to $35.6\text{ mg/kg bw per day}$). The NOAEL for systemic toxicity in rats was 75 mg/kg (equal to $2.7\text{ mg/kg bw per day}$), on the basis of a dose-related increase in the incidence of spongiosis hepatitis in males at 1000 and 2000 mg/kg . There was no evidence for significant long-term toxicity in females. Cyprodinil was not carcinogenic in mice or rats.

Cyprodinil gave negative results in a battery of studies of genotoxicity in bacteria and cultured mammalian cells in vitro, and in a test for micronuclei formation in mice in vivo.

The Meeting concluded that cyprodinil is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in rats and mice, the Meeting concluded that cyprodinil is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproduction in rats, reproductive parameters were not affected at the highest dose tested (4000 mg/kg , equal to $295\text{ mg/kg bw per day}$). The NOAEL for parental systemic toxicity was 1000 mg/kg (equal to $74\text{ mg/kg bw per day}$) on

the basis of decreased body-weight gain in F₀ females at the highest dose tested, 4000 mg/kg (equal to 295 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 mg/kg (equal to 74 mg/kg bw per day) on the basis of decreased body weights for F₁ and F₂ pups at the highest dose tested. Cyprodinil was not teratogenic in rats and rabbits at a dose of up to 1000 and 400 mg/kg bw per day in rats and rabbits, respectively. In a study of developmental toxicity in rats, lower fetal body weights and an increased incidence of delayed ossification at a dose of 1000 mg/kg bw per day were considered to be secondary to maternal toxicity. At the highest dose tested, a slight increase in the number of litters in which pups were born with an extra (thirteenth) rib was observed in rabbits in the presence of maternal toxicity, an effect that was not considered to be toxicologically relevant.

In a study of an acute neurotoxicity in rats, cyprodinil at a dose of 600 or 2000 mg/kg bw caused reduced activity, hunched posture, piloerection, increased responsiveness to stimuli, and hypothermia; the NOAEL was 200 mg/kg bw. In a study of short-term neurotoxicity, no signs of neurotoxicity were observed in a FOB, on evaluation of motor activity, or on neuropathological examination, in rats receiving cyprodinil in the diet at a concentration of up to 8000 mg/kg (equal to 601 mg/kg bw per day), the highest dose tested. The NOAEL was 800 mg/kg (equal to 54.5 mg/kg bw per day) on the basis of liver, kidney and thyroid histopathology, and reduced body-weight gain seen at the highest dose tested (8000 mg/kg, equal to 601 mg/kg bw per day).

Several soil and plant metabolites of cyprodinil were investigated using the Ames test and for acute oral toxicity at the highest dose (2000 mg/kg bw). The LD₅₀ for each of these metabolites was >2000 mg/kg bw and no mutagenic potential was detected.

The Meeting concluded that the existing data were adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg bw based on a NOAEL of 2.7 mg/kg bw per day in a 24-month study in rats fed with cyprodinil, on the basis of liver effects (spongiosis hepatitis) seen in males at higher doses, and a 100-fold safety factor.

The Meeting concluded that the establishment of an acute RfD for cyprodinil was not necessary, on the basis of its low acute toxicity, the absence of developmental toxicity in rats and rabbits, the lack of neurotoxicity after a single exposure, and absence of any other toxicological end-point that would be elicited by a single dose.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of toxicity and carcinogenicity ^a	Toxicity	2000 mg/kg, equal to 196 mg/kg bw per day	5000 mg/kg, equal to 558 mg/kg bw per day
		Carcinogenicity	5000 mg/kg, equal to 558 mg/kg bw per day ^c	—
Rat	2-year study of toxicity and carcinogenicity ^a	Toxicity	75 mg/kg, equal to 2.7 mg/kg bw per day	1000 mg/kg, equal to 35.6 mg/kg bw per day
		Carcinogenicity	2000 mg/kg, equal to 73.6 mg/kg bw per day ^c	
	Multi-generation study of reproductive toxicity ^a	Parental toxicity/offspring toxicity	1000 mg/kg, equal to 74.0 mg/kg bw per day	4000 mg/kg, equal to 295 mg/kg bw per day
	Study of developmental toxicity ^b	Maternal toxicity	200 mg/kg bw per day	1000 mg/kg bw per day
		Embryo- and fetotoxicity	200 mg/kg bw per day	1000 mg/kg bw per day
Rabbit	Study of acute neurotoxicity ^b	Neurotoxicity	200 mg/kg bw per day	600 mg/kg bw per day
Rabbit	Study of developmental toxicity ^b	Maternal toxicity	150 mg/kg bw per day	400 mg/kg bw per day
		Embryo- and fetotoxicity	400 mg/kg bw per day	—
Dog	1-year study of toxicity ^a	Toxicity	2500 mg/kg, equal to 66.0 mg/kg bw per day day	15 000 mg/kg, equal to 449 mg/kg bw per

^aDiet
^bGavage
^cHighest dose tested

Estimate of acceptable daily intake for humans

0–0.03 mg/kg bw

Estimate of acute reference dose

Unnecessary

Studies that would provide information useful for the continued evaluation of the compound

Further observations in humans.

Summary of critical end-points for cyprodinil

Absorption, distribution, excretion, and metabolism in mammals

Rate and extent of oral absorption	Rapid; maximum reached in blood by 0.15–1.0 h; about 71–85% absorbed after 48 h
Dermal absorption	At 6 µg/cm ² , absorption in rats in vivo was 21.7% in 0–24 h; at 870 µg/cm ² , absorption in vivo was 1.9% in 0–24 h
Distribution	Extensive; highest concentrations in liver, kidney, spleen, and blood
Potential for accumulation	No evidence of significant accumulation; about 0.2–0.6% of the total dose was found in tissues after 168 h
Rate and extent of excretion	Excretion was rapid; >90% excreted into urine (48–67%) and faeces (27–45%) within 48 h
Metabolism in animals	Very extensive; metabolic pathways include hydroxylation of the phenyl and pyrimidyl rings and conjugation with sulfate or glucuronic acid; limited cleavage of bond between phenyl and pyrimidyl rings; about 3–8% unchanged cyprodinil in faeces
Toxicologically significant compounds	Cyprodinil

Acute toxicity

Mouse, LD ₅₀ , oral	>5000 mg/kg bw
Rat, LD ₅₀ , oral	>2000 mg/kg bw
Rat, LD ₅₀ , dermal	>2000 mg/kg bw
Rat, LC ₅₀ , inhalation	>1.2 mg/l (maximum attainable concentration, 4-h exposure, nose only)
Rabbit, dermal irritation	Not an irritant
Rabbit, ocular irritation	Not an irritant
Skin sensitization	Sensitizing (maximization test)

Short-term studies of toxicity

Target/critical effect	Histopathological findings in liver, kidneys and thyroid
Lowest relevant oral NOAEL	19 mg/kg bw per day (90-day study in rats)
Lowest relevant dermal NOAEL	No suitable study is available
Lowest relevant inhalation NOAEC	No studies are available

Genotoxicity	No genotoxic potential
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Long-term studies of toxicity and carcinogenicity

Target/critical effect	Degenerative liver lesions (spongiosis hepatitis) in males, in rats
Lowest relevant NOAEL	2.7 mg/kg bw per day (2-year study in rats)
Carcinogenicity	No carcinogenicity in mice and rats

Reproductive toxicity

Reproduction target/critical effect	Reduced pup body weight
Lowest relevant reproductive NOAEL	74 mg/kg per day (rats)
Developmental target/critical effect	No toxicologically relevant effects were observed
Lowest relevant developmental NOAEL	400 mg/kg per day (rabbits)

Neurotoxicity/delayed neurotoxicity

Acute neurotoxicity	No evidence of neuropathology at doses of up to 2000 mg/kg bw in rats; NOAEL was 200 mg/kg bw, on the basis of clinical signs
90-day study of neurotoxicity	No evidence of neurotoxicity or neuropathology; NOAEL was 54.5 mg/kg bw per day on the basis of liver, kidney and thyroid histopathology

Other toxicological studies

Metabolites: study of acute toxicity	LD ₅₀ of >2000 mg/kg bw for four metabolites ^a
Metabolite: 90-day study, in diet	NOAEL of 79.5 mg/kg bw per day for CGA 249287
Metabolites: genotoxicity	No genotoxic potential for four metabolites ^a

Medical data	Limited data; slight eye irritation and sensitization reported in workers
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Summary	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	2-year study of toxicity and carcinogenicity	100
Acute RfD	Not allocated (unnecessary)	Not applicable	Not applicable

^a CGA 249287: (4-cyclopropyl-6-methyl-pyrimidine-2-ylamine)

CGA 275535: 3-(4-cyclopropyl-6-methyl-pyrimidine-2-ylamino)-phenol

NOA 422054: (4-cyclopropyl-6-hydroxymethyl-pyrimidine-2-ylamine)

CGA 321915: 4-cyclopropyl-6-methyl-pyrimidin-2-ol

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DIMETHOATE (addendum)

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Explanation

Dimethoate is an organophosphate ester, and virtually all its toxic effects are due to the inhibition of acetylcholinesterase activity. JMPR evaluated dimethoate for toxicological effects in 1963, 1965, 1967, 1984, 1987, and 1996. An acceptable daily intake (ADI) of 0–0.002 mg/kg bw was established in 1996 on the basis of the apparent NOAEL of 1.2 mg/kg bw per day for reproductive performance in a study of reproductive toxicity in rats and with a safety factor of 500. Although a safety factor of 100 would normally be used in deriving an ADI from a study of this type, the Meeting was concerned about the possibility that reproductive performance might have been affected at 1.2 mg/kg bw per day in this study and therefore used a higher-than-normal safety factor. No data were available to assess whether the effects on reproductive performance were secondary to the inhibition of cholinesterase activity. The 1996 JMPR concluded that it was not appropriate to base the ADI on the results of studies of volunteers, since the crucial end-point (reproductive performance) had not been assessed in humans. The present review was undertaken to consider the need for establishing an acute reference dose (RfD) and to evaluate new studies submitted by the sponsor.

Evaluation for acute reference dose

1. Neurotoxicity in rats

1.1 Acute neurotoxicity

(a) Administration by gavage

In a study that complied with the United States Environmental Protection Agency good laboratory practice (GLP) regulations, 40 CFR part 160, and guideline 81-8-SS, groups of 12 male and 12 female Sprague-Dawley Crl:CD BR rats (aged 43 days) were given dimethoate (purity, 99.1%) as a single dose at 0, 2 or 20 mg/kg bw by gavage in water. Additional groups of 15 male and 15 female rats received dimethoate at a dose of 200 mg/kg bw.

Observations and examinations were carried out until day 14 after dosing, when surviving animals were sacrificed. Viability, clinical signs and body weight were recorded. A functional observational battery (FOB) and motor activity evaluations were performed before treatment, at the time of peak effect, 2 h after dosing (as determined in a dose range-finding study), and on days 7 and 14. Brain weight and dimension were measured at termination. All animals were perfused in situ and neuropathological examinations were carried out on five randomly selected animals of each sex in the control group and in the group receiving a dose of 200 mg/kg bw.

No mortalities were observed during the study. A lower body-weight gain was measured in males over days 0–7 (–38%) at 200 mg/kg bw. The most remarkable clinical signs at this dose were observed on days 1 and/or 2 and included gait alterations (rocking, lurching or swaying), tremors (whole body or forelimbs/hindlimbs) and constricted pupils. Other clinical signs at 200 mg/kg bw consisted mainly of coloured material on the body and decreased defecation, and were observed on days 1, 2 and/or 3, but persisted until day 5 for one male. The most notable effects on FOB parameters in males and females were alterations in posture, convulsions, tremors and changes in faeces consistency during the home cage observations; lacrimation, salivation and changes in fur appearance during the handling observations; impaired mobility, gait alterations and decreased rearing activity during the open-field observations; alterations in approach, touch, startle, tail pinch and pupil responses, forelimb extension and air righting reflex during the sensory observations; reduced hindlimb extensor strength, reduced forelimb grip strength and impairments in rotarod performance during the neuromuscular observations; increased catalepsy times and decreased body temperature during the physiological observations at 200 mg/kg bw. Additionally, treatment-related reductions in mean ambulatory and total motor activity in males and females were apparent at this dose. The effects were noted approximately 2 h after treatment on day 0, and were transient in nature (gait alterations, tremors and constricted pupils persisted until days 1 and/or 2 for some animals), but on the basis of cage-side observations some symptoms persisted until day 5. None of the above signs were apparent on days 7 or 14. The only treatment-related effect on FOB parameters at 20 mg/kg bw was the absence of pupil response (5/12 males and 6/12 females affected versus 0/12 and 2/12 among the controls). No treatment-related changes in brain weights and dimensions or in central or peripheral nervous system tissues examined microscopically were observed at any dose. The presence of one female (of the five examined) at 200 mg/kg bw with minimally swollen sciatic nerve axons (none in concurrent control group) is not considered to be significant compared with the maximum incidence of 17% among historical controls.

The NOAEL for acute neurotoxicity in rats treated by gavage was 2 mg/kg bw on the basis of an absence of pupil response at ≥ 20 mg/kg bw (Lamb, 1993).

(b) *Administration in the diet*

Groups of 24 male and 24 female Sprague-Dawley Crl:CD BR VAF Plus rats (aged 8–9 weeks) received diet containing dimethoate (purity, 99.1%) providing a single nominal dose of 0, 1, 2, 3 or 15 mg/kg bw, in a study that complied with United States Environmental Protection Agency GLP regulations, 40 CFR part 160, and guideline 82-1. In a 2-week feeding adaptation phase before administration of the test material, the animals were conditioned to eat their daily ration of food over a short period of time. During this phase, animals received two-thirds of their daily food ration for 1 h between 06:00 and 07:00 and one-third of their daily ration of food for 1 h between 17:00 and 18:00. The test material was administered once on day 1 during the first daily feeding. Calculated intakes of dimethoate, which were close to the intended intakes, were 0, 0.96, 1.95, 2.94 and 15.01 mg/kg bw per day for males and 0, 0.98, 2.11, 3.23 and 15.01 mg/kg bw per day for females. For the remainder of the study, rats had access to control diet ad libitum until termination on day 15. Doses and time of peak effect (2.5–3 h after dosing) for FOB and/or detailed clinical observation, and for effects on cholinesterase activity were determined in two preliminary studies. Eight animals of each sex per dose were sacrificed for determination of cholinesterase activity (in plasma, erythrocytes and brain) on day 1, at time of peak effect. The remaining 16 animals of each sex per group were subjected to FOB and determination of locomotion activity on day 1 (time of peak effect) and on day 15. After these observations on day 15, all animals were sacrificed and determinations of cholinesterase activity were conducted on eight animals of each sex per group, and gross necropsy was conducted on the remaining eight animals of each sex per group. Clinical observations and determination of body weights and food consumption were made during the study. No histopathological examination was carried out. Determinations of cholinesterase activity (also measured before dosing in all animals) involved reaction with acetylthiocholine and 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB). The separated plasma and erythrocyte samples and brain sections were stored frozen at -70°C for up to 2 weeks if determination of cholinesterase activity was not possible at the day of sample collection.

There were no deaths or treatment-related clinical signs of toxicity and no effects on body weight or food consumption. FOB parameters and examination of motor activity showed no effects that could be attributed to the test material. Plasma cholinesterase activity was statistically significantly reduced on day 1 at 15 mg/kg bw in males (50% of control value) and in females (60% of control value); no significant reduction was observed on day 15. Erythrocyte cholinesterase activity was statistically significantly reduced on day 1 at 15 mg/kg bw in males (42% of control value) and in females (35% of control value) and at 3 mg/kg bw in males (71% of control value), while the reduction in males at 2 mg/kg bw (75% of control value) was not statistically significant. No significant differences were observed on day 15. Brain cholinesterase activity was significantly reduced on day 1 in both sexes at 15 mg/kg bw (hippocampus: 67% and 62% of control value in males and females, respectively; cortex: 64% and 57% of control value in males and females, respectively; striatum: 59% and 53% of control value in males and females, respectively) and in females at 3 mg/kg bw (cortex, 89% of control value). On day 15, inhibition of cholinesterase activity in the hippocampus was still statistically significant in both sexes at 15 mg/kg bw (84% and 85% of control value in males and females, respectively). There were no treatment-related effects on weight of the brain or on cholinesterase activity in the three regions of the brain investigated, and no macroscopic findings on days 1 or 15.

The NOAEL was 2 mg/kgbw on the basis of a statistically significant inhibition of cholinesterase activity in erythrocytes in males (29%) and in the brain cortex in females (11%) at ≥ 3 mg/kgbw. Recovery at 14 days after dosing appears to be incomplete at the highest dose of 15 mg/kgbw, since there was still a slight but statistically significant reduction of cholinesterase activity (15–16%) in the hippocampus (Schaefer, 1999).

1.2 Short-term study of neurotoxicity

In a study that complied with EPA GLP regulations, 40 CFR part 160, and guideline 82-7, groups of 10 male and 10 female (12 male and 12 female at the highest dose) Sprague-Dawley strain Crl:CD BR rats (aged 50 days) were given diets containing dimethoate (purity, 99.1%) at a concentration of 0, 1, 50 or 125 mg/kg for 91, 92, 93 or 94 days. These dietary concentrations provided intakes of 0, 0.06, 3.22 or 8.13 mg/kgbw per day for males and 0, 0.08, 3.78 or 9.88 mg/kgbw per day for females. Rats were randomly assigned to one of four study replicates to conduct FOB and tests for locomotor activity. Five rats of each sex from the control group and from the groups receiving the lowest and intermediate doses, and seven males and five females from the group receiving the highest dose were allocated for determination of plasma, erythrocyte and brain cholinesterase activities; the remaining five rats of each sex per group were allocated for neuropathological investigations. Clinical observations were recorded daily, body weights and food consumption were measured weekly, FOB and locomotor activity were assessed at weeks 3, 7 and 12. Cholinesterase activity was measured in plasma and erythrocytes before dosing and at weeks 3, 7 and 13, and in brain at week 13. Animals were not fasted before blood collection. Cholinesterase measurements in brain, and initial measurements in blood were performed according to the method of Dietz et al. (1973); this method uses the Ellman reaction, but the substrate is propionylthiocholine, not acetylthiocholine as proposed by Ellman et al. (1961). Later in the study, measurements of cholinesterase activity in erythrocytes and plasma were conducted at another laboratory by a method that used the Ellman reaction with acetylthiocholine as the substrate. All samples were stored at -20°C until analysis. Erythrocytes, plasma and brain from week 13 were stored for 3–7 days until analysis. Brains were weighed and dissected for measurement of brain cholinesterase activity at termination. Tissue from the central and peripheral nervous system was dissected and processed for histopathological examination after perfusion.

No mortalities occurred during the study. Small faeces in females at 50 mg/kg and in males and females at 125 mg/kg were noted primarily during weeks 2–6 of treatment. At 125 mg/kg, the mean body weights of males were slightly reduced during weeks 3–13, and cumulative mean body-weight gains were decreased in weeks 0–1 to 0–13. No treatment-related effects on food consumption, FOB (home cage, handling, open field, sensorimotor, neuromuscular and physiological observations) and locomotor activity (total and ambulatory motor activity) were apparent at any dose. Plasma cholinesterase activity was statistically significantly inhibited in males at 50 mg/kg (weeks 7 and 13) and at 125 mg/kg (weeks 3, 7 and 13). Erythrocyte cholinesterase activity was statistically significantly inhibited at week 7 and 13 in both sexes at 50 mg/kg (53% and 51% of control value in males; 66% and 58% in females) and at 125 mg/kg (40% and 47% of control value in males; 45% and 43% in females). At 1 mg/kg, erythrocyte cholinesterase activity in males was slightly, but statistically significantly reduced at week 7 (89% of control value), but no significant inhibition was seen at week 13. Statistically significant inhibition of cholinesterase activity was observed at 125 mg/kg in both sexes in the following brain regions: olfactory region (82% of control value in males), midbrain (85% of control value in males; 82% in

females), brainstem (83% of control value in males; 80% in females) and cortex (88% of control value in males). No differences were seen in absolute or relative brain and brain-region weights and no treatment-related effects were observed on neuropathological examination.

The NOAEL for systemic toxicity and neurotoxicity was 1 mg/kg (equal to 0.06 mg/kgbw per day) on the basis of inhibition of erythrocyte cholinesterase activity (34–49%) and small faeces at ≥ 50 mg/kg (equal to ≥ 3.22 mg/kgbw per day) (Lamb, 1994).

1.3 *Developmental neurotoxicity*

Groups of 24 pregnant female Crl:CD BR rats (aged 10–11 weeks) were given dimethoate (purity, 99.1%) at a dose of 0, 0.1, 0.5 or 3 mg/kgbw per day by oral gavage in water from day 6 of gestation until postnatal day 10; offspring received the same doses by oral gavage on postnatal days 11–21. This study complied with United Kingdom GLP regulations 1999, European Commission Directive 1999/11/EC, OECD GLP principles 1997, and EPA guideline OPPTS 870.6300. Clinical observation was performed on all dams at least twice daily throughout the study. Body weight and food consumption were recorded several times throughout the study; parturition and duration of gestation were noted. Ten dams per group were subjected to a detailed clinical examination and open arena observations on days 12 and 18 of gestation, and on postnatal days 4 and 10. On postnatal day 4, litters were culled to eight pups each (four males and four females when possible). Five pups from each litter were allocated to undergo further functional investigations (motor activity on postnatal days 13, 17, 22 and 59; auditory startle response habituation and pre-pulse inhibition of startle on postnatal days 23/24 and 60/61; learning and memory on postnatal days 23/24 and 61/62), while a sixth pup was sacrificed on postnatal day 11 for examination of the brain. In all litters except two in the group receiving a dose of 3 mg/kgbw per day, a different pup was allocated to each behavioural test. Physical development of the pups was assessed by measurement of body weight. Sexual maturation of female pups was assessed by age at vaginal opening, and maturation of males was assessed by age at balano-preputial separation. Dams were sacrificed on postnatal day 21 and subjected to gross necropsy, abnormal tissues being retained for possible histopathological examination. Groups of 10 male and 10 female offspring were selected for detailed neuropathological examination and sacrificed on postnatal day 21 or postnatal day 65 ± 2 , respectively.

Treatment of dams with a dose of 0.1, 0.5 or 3 mg/kgbw per day had no adverse effect on clinical condition, survival, body-weight gain or food intake during gestation and lactation, gestation length, macroscopic necropsy findings or brain weights. There was no evidence for neurotoxicity in the dams, according to functional observational battery assessments. There was no effect of treatment on mean implantation rate, litter size or on mean pup weights on postnatal day 1. At 3 mg/kgbw per day, all offspring in six litters showed signs of poor general condition or retarded development during early lactation. Three affected litters at 3 mg/kgbw per day and one litter at 0.5 mg/kgbw per day were killed on postnatal days 2–4 for reasons of animal welfare. In addition to these deaths of entire litters during early lactation, there was an increase in pup mortality among litters that survived to weaning in the group receiving a dose of 3 mg/kgbw per day: The number of pups found dead or that were killed up to postnatal day 21 was 15, 11, 24 and 44 for the control group and the groups receiving the low, intermediate and highest dose, respectively. Background data on controls from five studies in which littering took place between October 2000 and September 2002 showed that up to one litter was found dead or killed for reasons of animal

welfare and a range of 10 to 33 pups were missing, found dead or killed among litters surviving to weaning at postnatal day 21. At 3 mg/kg bw per day, body-weight gains of male and female offspring during postnatal days 1–4 were about 30% lower than those of controls, but the differences did not attain statistical significance. Thereafter, weight gains were comparable or only marginally inferior to those of controls, such that overall gains during postnatal days 1–21 were about 10% lower than those of controls. Direct dosing of offspring during postnatal days 11–21 did not adversely affect body-weight gains and no clinical signs were observed that were considered to be related to treatment. Among offspring maintained until postnatal day 65 ± 2 , there was no effect of treatment on general clinical condition, survival, body-weight gains during postnatal days 21–63 or age at attainment of sexual maturation. Treatment-related differences in the functional performance of offspring were limited to the groups receiving a dose of 3 mg/kg bw per day, before weaning. On postnatal day 4, males and female offspring tended to be less active than the controls, as shown by lower values for maximum pivoting angle, maximum distance travelled and number of sections entered in the arena. Although these differences did not achieve statistical significance, the consistency between all three measures, and between males and females, did indicate an effect of treatment. Reduced arena activity was also observed in males and females on postnatal day 21. There was no evidence of any treatment-related effect in offspring performance during post-weaning observations and functional testing, including monitoring of learning and memory, auditory startle response and sexual maturation. There was no effect of treatment on findings made on macroscopic necropsy or on brain weights of selected offspring killed on postnatal days 11, 21 or 65 ± 2 , or on brain length and width on postnatal days 21 and 65 ± 2 . Also, there was no effect of treatment on histopathological findings or brain morphometry for selected offspring killed on postnatal days 21 or 65 ± 2 .

Treatment with dimethoate at a dose of up to 3 mg/kg bw per day, the highest dose tested, was not associated with any selective developmental neurotoxicity. The NOAEL for functional development of the nervous system and systemic toxicity in the offspring was 0.5 mg/kg bw per day, on the basis of developmental delay in some functional parameters and increased pup mortality at a dose of 3 mg/kg bw per day, after maternal treatment by oral gavage from day 6 of gestation until postnatal day 10, and direct treatment of the offspring on postnatal days 11–21 (Myers, 2001c).

2. Effects on cholinesterase activity in rats

2.1 Study of cholinesterase activity after single and repeated doses of dimethoate

The purpose of this study, which complied with UK GLP regulations 1999, European Commission Directive 1999/11/EC and OECD GLP principles 1997, was to assess the effect of single and/or repeated doses of dimethoate (purity, 99.1%) on plasma, erythrocyte and brain cholinesterase activities in pregnant female Sprague-Dawley Crl:CD(SD)IGS BR rats and their pre-term fetuses, in pre-weaning offspring and in young adult rats. Recovery of cholinesterase activity was also assessed in young adults 39 days after cessation of treatment with repeated doses.

In trial A, groups of 19 mated female rats (aged 10–11 weeks) received dimethoate at a dose of 0, 0.1, 0.5 or 3 mg/kg bw per day by gavage in water. Ten females per group were treated from day 6 of gestation until postnatal day 10, while the remaining nine females were treated on days 6–20 of gestation. For the females treated until day 20 of gestation, eight dams per group were killed 3 h after dosing on that day; data on litters were assessed and cholinesterase activity was determined for maternal and fetal plasma, erythrocytes and

brain. For the females that were allowed to litter and were treated until postnatal day 10, offspring in eight litters per group were treated on postnatal days 11–21 inclusive, in order to assess effects on survival, body-weight gain and cholinesterase activity. Selected offspring from these litters were killed on postnatal day 4, 21 or 60, and cholinesterase activity was determined for plasma, erythrocytes and brain. Time of parturition and duration of gestation were noted. Body weight was noted several days during gestation and after parturition, until postnatal day 21.

In trial B, a group of eight pregnant female rats was not treated throughout the study. Of the offspring of these females, one male and one female per litter were given dimethoate as a single dose of 0, 0.1, 0.5 or 3 mg/kg bw by gavage in water on postnatal day 11. Eight male and eight female offspring per group (one male and one female from each litter) were killed 2 h after dosing and cholinesterase activity was determined for plasma, erythrocytes and brain.

In trial C, groups of 16 male and 16 female naive adult rats (aged 7–8 weeks) received dimethoate at a dose of 0, 0.1, 0.5 or 3 mg/kg bw per day by gavage in water. Eight males and eight females per group were treated for one day and were killed 2 h after dosing, while the remaining eight males and eight females per group were treated for 11 consecutive days, and killed 2 h after the last dose. In each case, cholinesterase activity was determined for plasma, erythrocytes and brain.

Blood samples were collected from the retro-orbital sinus (for pups on postnatal day 21 and adults), umbilical cord (fetuses on day 20 of gestation) or by decapitation (pups on postnatal day 4 or 11). Blood samples were taken 3 h after dosing on day 20 of gestation, 4 h after dosing on postnatal day 4, and 2 h after dosing on postnatal days 11–21. Fetal blood samples from day 20 of gestation were pooled for each litter. Plasma samples and erythrocyte haemolysates were stored at -80°C until analysis. Brains were removed immediately after sacrifice, weighed and frozen in liquid nitrogen. Fetal brains from day 20 of gestation were pooled for each litter because the range-finding study showed no difference in cholinesterase activity between the sexes. Cholinesterase activity was determined according to a modified Ellman method (Environmental Protection Agency, 1999). Erythrocyte cholinesterase activity was measured using 6-6'-dithiodinicotinic acid, plasma and brain activity was measured using 5,5-dithio-bis(2-nitrobenzoic acid) as colour component.

On day 20 of gestation, the dams were sacrificed immediately after blood sampling, and brains were collected. The reproductive tract was examined for the following parameters: number of corpora lutea, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each horn. Fetuses were weighed and sexed, and sacrificed on a cool plate at 0°C . The brains of the fetuses were then sampled. The dams were thoroughly examined macroscopically and specimens of any abnormal tissue were retained. All pups (except those culled on postnatal day 4) were examined macroscopically. In order to gain experience in a specific method of whole body perfusion fixation of tissue, and in precise sectioning of brains from young adult rats, five offspring were killed on postnatal day 61, perfused and the brain embedded, sectioned and subjected to examination by light microscopy.

There were no clinical signs that were considered to be related to treatment and no deaths at any dose. There was no adverse effect of treatment on weight gain of adult males

and females, weight change of dams during gestation and lactation or macroscopic findings at necropsy. Litter data on day 20 of gestation and body weight, weight gain and survival of offspring up to postnatal day 11 were unaffected by treatment. There was no effect of direct treatment on the growth or survival of the offspring.

In trial A, on day 20 of gestation, plasma and erythrocyte cholinesterase activity was statistically significantly inhibited at 3 mg/kg bw per day in dams (56% and 42% of control value) and in fetuses (57% and 69% of control value). Brain cholinesterase activity was statistically significantly inhibited in dams at 0.5 and 3 mg/kg bw per day (90% and 40% of control value) and in fetuses at 0.1, 0.5 and 3 mg/kg bw per day (88%, 90% and 67% of control value). In pups at postnatal day 4, plasma cholinesterase activity was statistically significantly inhibited at 0.5 and 3 mg/kg bw per day in females (92% and 90% of control value), while erythrocyte cholinesterase activity was statistically significantly inhibited at 3 mg/kg bw per day in males only (83% of control value). Brain cholinesterase activity was statistically significantly inhibited in males at 0.1, 0.5 and 3 mg/kg bw per day (90%, 92% and 87% of control value). In pups on postnatal day 21, plasma cholinesterase activity was statistically significantly inhibited at 3 mg/kg bw per day in both sexes (61% of control value in males; 62% in females), while erythrocyte cholinesterase activity was statistically significantly inhibited at 3 mg/kg bw per day in both sexes (41% of control value in males; 35% in females) and at 0.5 mg/kg bw per day in females (77% of control value). Brain cholinesterase activity was statistically significantly inhibited in males at 0.1, 0.5 and 3 mg/kg bw per day (96%, 87% and 55% of control value) and in females at 0.5 and 3 mg/kg bw per day (88% and 58% of control value). Offspring killed on postnatal day 60 (i.e. 39 days after the end of repeated dosing) showed complete recovery of cholinesterase activity; the statistically significant inhibition of brain cholinesterase activity in females at 0.5 and 3 mg/kg bw per day (96% and 96% of control value) was considered to be not biologically significant.

In trial B, in offspring treated once on postnatal day 11, plasma and brain cholinesterase activity was statistically significantly inhibited at 3 mg/kg bw per day in males (81% and 83% of control value). In females, inhibition of plasma, erythrocyte and brain cholinesterase activities was seen at the same dose (82%, 74% and 82% of control value), differences attaining statistical significance for brain cholinesterase activity only. The statistically significant inhibition of brain cholinesterase activity in males at 0.5 mg/kg bw per day (95% of control value) was considered to be not biologically significant.

In trial C, treatment of naive adult animals with a single dose of 3 mg/kg bw per day was associated with inhibition of plasma, erythrocyte and brain cholinesterase activity in males (81%, 83% and 88% of controls) and in females (87%, 73% and 86% of controls); differences attained statistical significance for all parameters in males and for erythrocyte and brain cholinesterase activity in females. The statistically significant inhibition of brain cholinesterase activity in males at 0.5 mg/kg bw per day (96% of control value) was considered to be not biologically significant. Treatment of naive adults with 11 consecutive doses of dimethoate at 3 mg/kg bw per day was associated with inhibition of plasma, erythrocyte and brain cholinesterase activity in males (63%, 42% and 53% of controls) and in females (79%, 37% and 42% of controls); differences attained statistical significance for all parameters, except plasma cholinesterase activity in females. At 0.5 mg/kg bw per day, there was a slight, statistically non-significant inhibition of plasma and erythrocyte cholinesterase activity in males (88% and 83% of controls), while brain

cholinesterase activity was statistically significantly inhibited in males and females (90% and 87% of controls).

The NOAEL for dimethoate given as a single dose was 0.5 mg/kg bw per day on the basis of a statistically significant inhibition of brain cholinesterase activity in pre-weaning rats (17–18%) and in young adults (12–14%) and a significant inhibition of erythrocyte cholinesterase activity in pre-weaning females (26%) and in young adult females (27%) at 3 mg/kg bw per day.

The NOAEL for dimethoate given as repeated doses was 0.1 mg/kg bw per day on the basis of a slight, statistically significant inhibition (10–13%) of brain cholinesterase activity in pregnant rats, pups on postnatal day 21 and young adult rats, and a statistically significant inhibition (23%) of erythrocyte cholinesterase activity in female pups on postnatal day 21 at ≥ 0.5 mg/kg bw per day. The slight, statistically significant depression (10–12%) of brain cholinesterase activity at 0.1 and 0.5 mg/kg bw per day in fetuses and in male pups aged 4 days was considered to be of doubtful biological significance, since there was no dose–response relationship (Myers, 2001a).

2.2 *Dose-finding study preliminary to study of developmental neurotoxicity*

Groups of 15 pregnant female Crl:CD BR rats (aged 10–11 weeks) were given dimethoate (purity, 99.1%) at a dose of 0, 0.2, 3 or 6 mg/kg bw per day by gavage in water. This study did not comply with GLP as it was not audited by quality assurance (QA). Ten females per group were treated from day 6 of gestation until postnatal day 10 and the remaining five females per group were treated on days 6–20 of gestation. The females treated until day 20 of gestation were killed 3 h after dosing on that day; data on litters were assessed and cholinesterase activity was determined in maternal and fetal plasma, erythrocytes and brain. For the females that were allowed to litter and were treated until postnatal day 10, two male and two female offspring per litter were treated on postnatal days 11–21, in order to assess effects on survival, weight gain and cholinesterase activity in plasma, erythrocytes and brain. The treated offspring were killed 2 h after dosing on postnatal day 21 and cholinesterase activity was determined. The remaining offspring in each litter were not treated and acted as within-litter “controls” for comparison of growth and survival among siblings. Dams and untreated offspring were killed on or shortly after postnatal day 21. In order to gain experience in gravity perfusion and histological sectioning of brains from pups on postnatal day 21 to allow examination of specific regions of the brain, four untreated pups were killed on postnatal day 21, perfused and the brain embedded, sectioned and subjected to examination by light microscopy. For cholinesterase measurements, blood samples were taken from the retro-orbital sinus (adults and pups) and umbilical cord (fetuses) 2–3 h after dosing. The fetal blood samples from day 20 of gestation were pooled for each litter. The resulting plasma samples and erythrocyte haemolysates were stored at -80°C until analysis. The brains were removed immediately after sacrifice, weighed and frozen in liquid nitrogen and stored until analysis. The fetal brains from day 20 of gestation were pooled for males and females fetuses for each litter. Cholinesterase activity was determined according to a modification of the Ellman method (Environmental Protection Agency, 1996).

In the dams, no deaths occurred throughout the study, and no differences in clinical signs were detected between groups. Body weight was affected at a dose of 6 mg/kg bw per day and 3 mg/kg bw per day. A statistically significantly reduced maternal body-weight gain

was observed on days 10–20 of gestation at 6 mg/kg bw per day and at 3 mg/kg bw per day. Lower (but not statistically significantly different from control) mean body weight that persisted until postnatal day 17 was noted at both doses. Food consumption was not affected by treatment. No treatment-related findings were noted at necropsy on day 20 of gestation or postnatal day 21. In the dams killed on day 20 of gestation, cholinesterase activity was inhibited at 3 and at 6 mg/kg bw per day in plasma (75% and 43% of control value), in erythrocytes (22% and 15% of control value) and in brain (25% and 12% of control value).

Litter parameters on day 20 of gestation and the number of implantation sites were unaffected by the treatment. In the fetuses, plasma cholinesterase activity was inhibited at 0.2, 3 and 6 mg/kg bw per day in males (80%, 25% and 21% of control value) and females (88%, 34% and 27% of control value). Erythrocyte cholinesterase activity was inhibited in males at 3 and 6 mg/kg bw per day (30% and 13% of control value) and in females at 0.2, 3 and 6 mg/kg bw per day (70%, 18% and 4% of control value). Brain cholinesterase activity was inhibited at 3 and 6 mg/kg bw per day in males (78% and 65% of control value) and females (76% and 58% of control value). Total litter size on postnatal day 1 was not affected by treatment. However, increased postnatal mortality was observed at 6 mg/kg bw per day. Two normal-sized litters died on postnatal day 2 and postnatal day 5, while another dam lost 4/14 offspring on postnatal day 4. This resulted in reductions in live litter size and offspring viability index. Group mean body weight and body-weight gain for the offspring was reduced during postnatal days 1–11 at 6 mg/kg bw per day. Direct dosing of offspring on postnatal days 11–21 did not affect offspring survival, body weight or body-weight gain at any dose. On postnatal day 21, cholinesterase activity was inhibited at 3 and at 6 mg/kg bw per day in plasma (61% and 40% of control value in males, 60% and 40% of control value in females, respectively), in erythrocytes (40% and 30% of control value in males, 35% and 20% of control value in females, respectively) and in brain (55% and 45% of control value in males, 58% and 34% of control value in females, respectively).

The NOAEL was 0.2 mg/kg bw per day for dams and their offspring under the conditions of this study, on the basis of reduced body-weight gain in dams and inhibition of erythrocyte and brain cholinesterase activity in dams, fetuses and pups at 3 mg/kg bw per day. The slightly decreased erythrocyte cholinesterase activity in female fetuses at 0.2 mg/kg bw per day was considered to be not biologically significant, since there were no effects on brain cholinesterase activity nor any effects in males (Myers, 2001b).

2.3 Study of developmental neurotoxicity

Groups of 24 pregnant female Crl:CD BR rats (aged 10–11 weeks) were given dimethoate (purity, 99.1%) by oral gavage in water at a dose of 0, 0.1, 0.5 or 3 mg/kg bw per day from day of gestation until postnatal day 10; offspring received the same doses by oral gavage on postnatal days 11–21. This study complied with UK GLP regulations 1999, European Commission Directive 1999/11/EC, OECD GLP principles 1997, and EPA guideline OPPTS 870.6300. Clinical observation was performed on all dams at least twice daily throughout the study. Body weight and food consumption were recorded several times throughout the study; parturition and duration of gestation were noted. Ten dams per group were subjected to a detailed clinical examination and open arena observations on days 12 and 18 of gestation, and on postnatal days 4 and 10. On postnatal day 4, litters were culled to eight pups each (four males and four females when possible). Five pups from each litter

were allocated to undergo further functional investigations (motor activity on postnatal days 13, 17, 22 and 59; auditory startle response habituation and pre-pulse inhibition of startle on postnatal days 23/24 and 60/61; learning and memory on postnatal days 23/24 and 61/62), while a sixth pup was sacrificed on postnatal day 11 for examination of the brain. In all litters except two in the group receiving a dose of 3 mg/kg bw per day, a different pup was allocated to each behavioural test. Physical development of the pups was assessed by measurement of body weight. Sexual maturation of female pups was assessed by age at vaginal opening, and maturation of males was assessed by age at balano-preputial separation. Dams were sacrificed on postnatal day 21 and subjected to gross necropsy, abnormal tissues being retained for possible histopathological examination. Groups of 10 male and 10 female offspring were selected for detailed neuropathological examination and sacrificed on postnatal day 21 or postnatal day 65 ± 2 , respectively.

Treatment of dams with a dose of 0.1, 0.5 or 3 mg/kg bw per day had no adverse effect on clinical condition, survival, body-weight gain or food intake during gestation and lactation, gestation length, macroscopic necropsy findings or brain weights. There was no evidence for neurotoxicity in the dams, according to functional observational battery assessments. There was no effect of treatment on mean implantation rate, litter size or on mean pup weights on postnatal day 1. At 3 mg/kg bw per day, all offspring in six litters showed signs of poor general condition or retarded development during early lactation. Three affected litters at 3 mg/kg bw per day and one litter at 0.5 mg/kg bw per day were killed on postnatal days 2–4 for reasons of animal welfare. In addition to these deaths of litters during early lactation, there was an increase in pup mortality among litters that survived to weaning in the group receiving a dose of 3 mg/kg bw per day: The number of pups found dead or that were killed up to postnatal day 21 was 15, 11, 24 and 44 for the control group and the groups receiving the low, intermediate and highest dose, respectively. Background data on controls from five studies in which littering took place between October 2000 and September 2002 showed that up to one litter was found dead or killed for reasons of animal welfare and a range of 10 to 33 pups were missing, found dead or killed among litters surviving to weaning at postnatal day 21. At 3 mg/kg bw per day, body-weight gains of male and female offspring during postnatal days 1–4 were about 30% lower than those of controls, but the differences did not attain statistical significance. Thereafter, weight gains were comparable or only marginally inferior to those of controls, such that overall gains during postnatal days 1–21 were about 10% lower than those of controls. Direct dosing of offspring during postnatal days 11–21 did not adversely affect body-weight gains and no clinical signs were observed that were considered to be related to treatment. Among offspring maintained until postnatal day 65 ± 2 , there was no effect of treatment on general clinical condition, survival, body-weight gains during postnatal days 21–63 or age at attainment of sexual maturation. Treatment-related differences in the functional performance of offspring were limited to the groups receiving a dose of 3 mg/kg bw per day, before weaning. On postnatal day 4, males and female offspring tended to be less active than the controls, as shown by lower values for maximum pivoting angle, maximum distance travelled and number of sections entered in the arena. Although these differences did not achieve statistical significance, the consistency between all three measures, and between males and females, did indicate an effect of treatment. Reduced arena activity was also observed in males and females on postnatal day 21. There was no evidence of any treatment-related effect in offspring performance during post-weaning observations and functional testing, including monitoring of learning and memory, auditory startle response and sexual maturation. There was no effect of treatment on findings made on macroscopic necropsy or on brain weights of selected offspring killed on postnatal days 11, 21 or 65 ± 2 , or on brain

length and width on postnatal days 21 and 65 ± 2 . Also, there was no effect of treatment on histopathological findings or brain morphometry for selected offspring killed on postnatal days 21 or 65 ± 2 .

Treatment with dimethoate at a dose of up to 3 mg/kgbw per day, the highest dose tested, was not associated with any selective developmental neurotoxicity. The NOAEL for functional development of the nervous system and systemic toxicity in the offspring was 0.5 mg/kgbw per day, on the basis of developmental delay in some functional parameters and increased pup mortality at a dose of 3 mg/kgbw per day, after maternal treatment by oral gavage from day 6 of gestation until postnatal day 10, and direct treatment of the offspring on postnatal days 11–21 (Myers, 2001c).

3. Studies on metabolites

3.1 *Effects of dimethoate, omethoate and four metabolites on erythrocyte cholinesterase activity*

In a study that was performed according to UK GLP regulations for 1999, European Commission Directive 1999/11/EC and OECD GLP principles for 1997, groups of five male Crl:CD(SD)IGS BR rats (aged 8 weeks) received one of the following treatments: (i) dimethoate (purity, 99.5%) in a single dose of 30 mg/kgbw; or (ii) four metabolites (metabolite I: *O*-desmethyl omethoate potassium salt (purity, 98.6%); metabolite II: *O*-desmethyl omethoate carboxylic acid potassium salt (purity, 89.5%); metabolite III: *O*-desmethyl-*N*-desmethyl omethoate potassium salt (purity, 96.4%); metabolite IV: *O*-desmethyl-isodimethoate dicyclohexylammonium salt (purity, 98.2%) as a single dose of 30 mg/kgbw; or (iii) omethoate (purity, 96.3%) as a single dose of 5 mg/kgbw, given by oral gavage in water.

Detailed clinical observations were made before dosing, at return to home cage, after dosing of the group and every 2 h for the first 24 h after dosing of the group. Body-weight was recorded before dosing, on the day of treatment and twice weekly thereafter. Blood samples for determination of erythrocyte cholinesterase activity were taken before dosing (after a fast of 8 h), at about 2.5 h after dosing and 24 h after dosing (again after 8 h fasting). All animals were killed at termination and examined macroscopically.

No mortalities, no clinical signs of toxicity and no effects on body-weight gain were observed in any group during 14 days. No treatment-related findings were noted at necropsy. Markedly and statistically significantly lower erythrocyte cholinesterase activity was seen for animals receiving dimethoate at a dose of 30 mg/kgbw (47% and 60% of the pre-dosing value at 2.5 h and 24 h after treatment, respectively) or omethoate at a dose of 5 mg/kgbw (26% and 66% of the pre-dosing value at 2.5 h and 24 h after treatment, respectively). For animals receiving metabolites I, II or IV, erythrocyte cholinesterase activity decreased slightly at 2.5 h after dosing (81%, 75% or 72% of pre-dosing value) and at 24 h after dosing (84%, 79% or 80% of the pre-dosing value). No effect on erythrocyte cholinesterase activity was seen in animals receiving metabolite III (Brennan, 2001).

3.2 *Peak effect of omethoate on clinical signs*

Groups of five male and five female Wistar Crl Glx Brl Han:WI rats (aged about 7 weeks) received omethoate (purity, 96.5%) by oral gavage in water as a single dose of 0, 5, 10 or 15 mg/kgbw, in a study that did not comply with GLP as it was not audited by QA.

Detailed clinical observations (including abbreviated FOB) were made before treatment, immediately after dosing and at 1, 2, 4, 7 and 24 h thereafter. Body-weight was determined once before dosing. Blood samples for the determination of plasma and erythrocyte cholinesterase activity were taken from the retro-orbital sinus 8 h after dosing and before necropsy (24 h after dosing). Brains were removed immediately after sacrifice for determination of cholinesterase activity; no further pathology examinations were carried out. Cholinesterase activity was determined according to a modified Ellman method (Environmental Protection Agency, 1996). Erythrocyte cholinesterase activity was measured using the colour component DNTA, while plasma and brain cholinesterase activity was measured using DNTB.

No animals died during the study. Major clinical signs of cholinergic stimulation that occurred in a dose-dependent and time-dependent manner in the treated groups were tremors, impairment of gait, loss of pupillary reflex, irregular respiration, and frequent chewing. The time of peak effect was 2–4 h at the lowest dose and 1–4 h at the intermediate and highest dose. Plasma cholinesterase activity was statistically significantly inhibited in males at 5, 10 and 15 mg/kg bw (48%, 41% and 36% of control value after 8 h; 68%, 52% and 47% of control value after 24 h) and in females at 10 and 15 mg/kg bw (48% and 53% of the control value after 8 h; 52% and 45% of the control value after 24 h). Erythrocyte cholinesterase activity was statistically significantly inhibited in both sexes at 5, 10 and 15 mg/kg bw (males: 44%, 35% and 26% of the control value after 8 h, and 55%, 37% and 28% of the control value after 24 h; females: 41%, 32% and 31% of the control value after 8 h, and 51%, 36% and 30% of the control value after 24 h). Brain cholinesterase activity was inhibited in both sexes at 5, 10 and 15 mg/kg bw (males: 59%, 44% and 41% of the control value; females: 60%, 56% and 44% of the control value), the differences attained statistical significance for males at the highest dose and for females at the intermediate and highest doses (Mellert et al., 2002a).

3.3 *Inhibition of cholinesterase activity by omethoate*

Groups of 10 male Wistar Crl Glx Brl Han:WI rats (aged about 7 weeks) received omethoate (purity, 96.5%) as a single dose of 0, 0.25, 0.5, 0.75 or 1.5 mg/kg bw by oral gavage in water, in a study that did not comply with GLP as it was not audited by QA. A check for moribund or dead animals was made twice daily, and body weight was determined once before dosing. Blood samples for determination of plasma and erythrocyte cholinesterase activities were taken from the retro-orbital sinus 2.5 h after dosing and before necropsy (24 h after dosing). Brains were removed immediately after sacrifice for the determination of cholinesterase activity; no further pathology examinations were carried out. Cholinesterase activity was determined according to a modified Ellman method (Environmental Protection Agency, 1996). Erythrocyte cholinesterase activity was measured using the colour component DNTA, while plasma and brain cholinesterase activity was measured using DNTB.

No animals died and no abnormal clinical signs were detected during the study. Plasma cholinesterase activity was statistically significantly inhibited at 0.75 and 1.5 mg/kg bw at 2.5 h after dosing (81% and 65% of control value). Erythrocyte cholinesterase activity was statistically significantly inhibited at 0.25, 0.5, 0.75 and 1.5 mg/kg bw at 2.5 h after dosing (87%, 73%, 62% and 44% of the control value) and at 0.75 and 1.5 mg/kg bw at 24 h after dosing (86% and 75% of the control value). Brain cholinesterase activity was marginally inhibited at 0.5 and 0.75 mg/kg bw and moderately inhibited at 1.5 mg/kg bw

(87%, 89% and 69% of control value); the differences did not attain statistical significance (Mellert et al., 2002b).

Comments

The LD₅₀ of dimethoate administered orally was about 314–600 mg/kg bw in rats and 150 mg/kg bw in mice.

Acute neurotoxicity was studied in rats given dimethoate at a single dose of 0, 2, 20 or 200 mg/kg bw by gavage, after preliminary studies had shown that peak effects for clinical signs occur about 2 h after dosing. Abnormal clinical signs and effects on FOB parameters were seen at the highest dose, mainly during the first 2 days after treatment and disappeared by day 7. The NOAEL was 2 mg/kg bw on the basis of the absence of pupil response at ≥ 20 mg/kg bw. Cholinesterase activity was not analysed in this study.

In a study of acute neurotoxicity in rats given dimethoate in the diet as a single doses of 0, 1, 2, 3 or 15 mg/kg bw, no clinical signs and no effects on FOB parameters were observed. A statistically significant inhibition of cholinesterase activity in erythrocytes of males (29%) and in the brain cortex of females (11%) was observed at ≥ 3 mg/kg bw.

In a special study designed to assess effects on cholinesterase activity, pre-weaning rats (aged 11 days) and young adult rats (aged 7–8 weeks) received dimethoate as a single dose of 0, 0.1, 0.5 or 3 mg/kg bw by gavage. There was no difference in susceptibility between pre-weaning and young adult rats. A statistically significant inhibition of brain cholinesterase activity in pre-weaning rats (17–18%) and in young adult rats (12–14%) and of erythrocyte cholinesterase activity in pre-weaning and young adult female rats (26–27%) was observed at 3 mg/kg bw.

The Meeting concluded that the overall NOAEL for acute effects on cholinesterase activity was 2 mg/kg bw.

The Meeting also considered a number of studies in human volunteers, which indicated that single or repeated oral doses of dimethoate of up to 0.2 mg/kg bw did not induce clinical effects or inhibit cholinesterase activity in the blood. It was concluded that these studies were not conducted according to current standards (no details on study design, e.g. age and sex of individual volunteers, were given and no raw data were provided). Therefore, the Meeting considered that the studies in humans were only supportive for setting the acute RfD.

The Meeting also reviewed new studies that were not relevant to the establishment of an acute RfD. In a study of neurotoxicity, rats received dimethoate in the diet at concentrations of 0, 1, 50 or 125 mg/kg. The NOAEL for systemic toxicity and neurotoxicity was 1 mg/kg (equal to 0.06 mg/kg bw per day) on the basis of inhibition of erythrocyte cholinesterase activity (34–49%) and small faeces at ≥ 50 mg/kg (equal to ≥ 3.22 mg/kg bw per day).

In a special study designed to assess effects on cholinesterase activity, pregnant rats, pre-weaning rats and young adult rats received dimethoate by gavage as repeated doses at 0, 0.1, 0.5 or 3 mg/kg bw per day. The NOAEL was 0.1 mg/kg bw per day on the basis of a

consistent, statistically significant inhibition of brain cholinesterase activity (10–13%) in pregnant, pre-weaning and young adult rats and of erythrocyte cholinesterase activity (23%) in pre-weaning female pups at ≥ 0.5 mg/kg bw per day.

In a study of developmental neurotoxicity, pregnant rats received dimethoate by gavage at a dose of 0, 0.1, 0.5 or 3 mg/kg bw per day from day 6 of gestation to postnatal day 10, and their offspring received the same doses by gavage on postnatal days 11–21. The NOAEL for functional development of the nervous system and systemic toxicity in the offspring was 0.5 mg/kg bw per day on the basis of developmental delay in some functional parameters and increased pup mortality at a dose of 3 mg/kg bw per day. The Meeting considered these effects to be of no relevance for setting the acute RfD, since they would not be expected to occur after a single exposure, and concluded that the new studies supported the current ADI of 0–0.002 mg/kg bw.

Toxicological evaluation

After considering the previous evaluations of dimethoate and the new data submitted, the Meeting established an acute RfD of 0.02 mg/kg bw on the basis of the overall NOAEL of 2 mg/kg bw for cholinesterase inhibition in studies in rats, and a safety factor of 100. This acute RfD was supported by the NOAEL of about 0.2 mg/kg bw per day in studies in volunteers receiving single or repeated doses, which were evaluated by the 1996 JMPR.

The Meeting recognized that it might be possible to refine this acute RfD on the basis of further characterization of the effects caused by dimethoate.

Estimate of acute reference dose

0.02 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

- Further observations in humans
- The two-generation study of reproductive toxicity (available in abbreviated form at the 2003 Meeting)

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FAMOXADONE

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Famoxadone and its metabolites in rats and dogs	

Explanation

Famoxadone is the ISO approved common name for 5-methyl-5-(4-phenoxyphenyl)-3-phenylamino-2,4-oxazolidinedione. It is a racemic mixture containing two enantiomers in a 50:50 ratio.

Famoxadone has not been evaluated previously by the JMPR. Consequently, famoxadone was reviewed at the present Meeting in the context of the JMPR New Compounds Review Programme.

Famoxadone is used in agriculture, viticulture and horticulture for the control of a wide range of key fungal diseases of grapes, tomatoes and cereals. It inhibits mycelial growth and zoospore survival of various Oomycete fungi, e.g. *Plasmopara viticola* that causes grape downy mildew and *Phytophthora infestans* that causes tomato late blight, including mating types A1 and A2 that are resistant to phenylamide fungicides. Mycelial

growth and/or spore germination of non-Oomycetes are also inhibited. Sensitive species encountered as cereal pathogens include *Septoria* spp., eyespot, brown rust, yellow rust and powdery mildew in wheat and net blotch, *Rhynchosporium*, brown rust and powdery mildew in barley.

The mechanism of antifungal action of famoxadone is inhibition of the mitochondrial respiratory chain at complex III, which results in decreased production of ATP.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

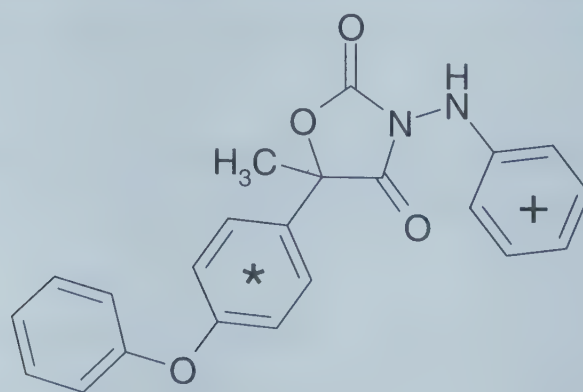
The positions of the radiolabel in the compounds used in the studies of absorption, distribution and excretion are shown in Figure 1 and the metabolic pathways of famoxadone in rats are shown in Figure 2.

Rats

The absorption, distribution, and excretion of famoxadone [5-methyl-5-(4-phenoxyphenyl)-3-phenylamino-2,4-oxazolidinedione] was evaluated in male and female CrI:CD BR (Sprague-Dawley) rats given famoxadone as a single oral dose at either 5 or 100 mg/kg bw, or as repeated doses at 5 mg/kg bw per day for 14 days. Pharmacokinetic and metabolic parameters were examined using [^{14}C -phenoxyphenyl (POP)]famoxadone and [^{14}C -phenylamino (PA)]famoxadone. The stereoselective metabolism of the two enantiomers of famoxadone was also investigated at the two doses (Savides et al., 1995; Savides et al., 1996; Himmelstein, 1999a).

Male and female CrI:CD®BR (Sprague-Dawley) rats (four or five rats of each sex per treatment, except in a pilot study in which two rats of each sex were used) were given the radiolabelled test material orally by gavage as a suspension in sodium carboxymethyl cellulose (1%) and ammonium acetate (0.01 mmol/l). Rats received either [^{14}C -POP]famoxadone (radiochemical purity, >99%) or [^{14}C -PA]famoxadone (radiochemical purity, >98%). In addition, some rats were treated with unlabelled famoxadone for 14 days before

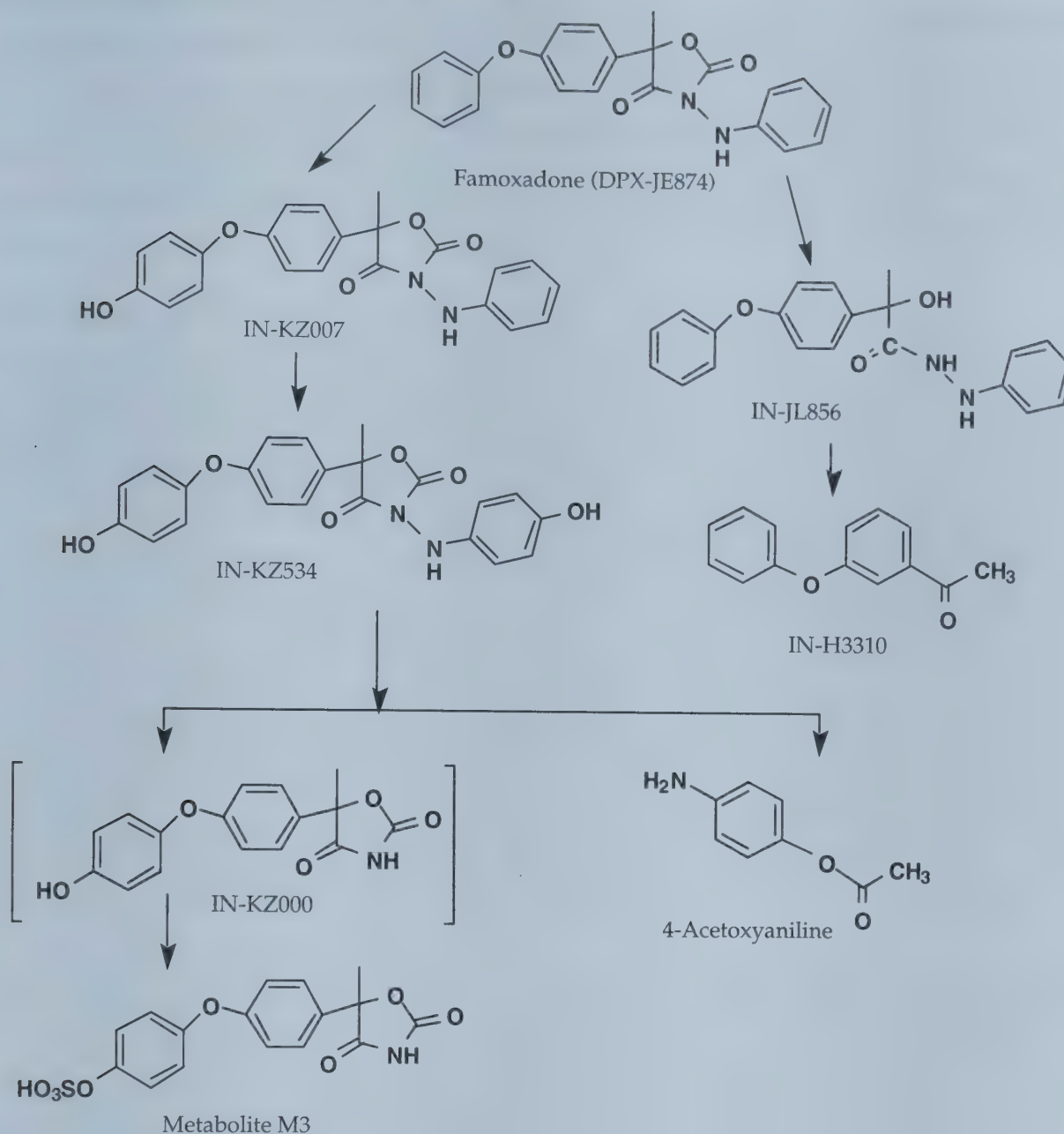
Figure 1. Positions of radiolabel on famoxadone used in studies of metabolism



* denotes [^{14}C -phenoxyphenyl]famoxadone

+ denotes [^{14}C -phenylamino]famoxadone

Figure 2. Proposed metabolic pathway of famoxadone in rats



receiving [^{14}C -PA]famoxadone. Pilot studies showed that a termination time of 120 h was sufficient to ensure that 90% of the administered radioactive dose was excreted. The initial absorption of famoxadone was rapid. The mean apparent absorption half-lives of total radioactive residues in the whole blood and plasma were approximately 0.8–1.4 h after an oral dose of 5 mg/kg bw of [^{14}C -PA]famoxadone, or 100 mg/kg bw of [^{14}C -POP]famoxadone. At a dose of 100 mg/kg bw, the mean apparent absorption time for [^{14}C -PA] was 3.9–6.9 h. This difference suggests rapid enteric metabolism involving cleavage of the phenylamino group from the remainder of the molecule, and more rapid absorption of the phenoxyphenyl residues. Also, the rate of absorption of the phenylamino residue was saturable at a high dose. Excretion was rapid, with almost all the administered dose being recovered from the faeces (>75% within 24 h after dosing, and >90% within 120 h). In general, urinary elimination accounted for <10% of the administered dose. Pretreatment of male and female rats with unlabelled famoxadone had no significant effect on the rates of excretion of [^{14}C -PA]famoxadone residues in either the faeces or urine. [^{14}C -POP]famoxadone was not examined in this way in rats (but was in mice, see below). Expired air did not contain $^{14}\text{CO}_2$. The ratios of radiolabel in tissue: blood were <1. Although there was rapid elimination of [^{14}C -PA]famoxadone from plasma, the half-lives for elimination of [^{14}C -PA]famoxadone

equivalent residues from erythrocytes were approximately two- to three-fold longer. It appeared, therefore, that the radiolabelled residues from the phenylamino moiety were binding to erythrocytes. No binding of [¹⁴C-POP]famoxadone equivalent residues was observed in either whole blood or plasma. Plasma half-lives were about 7 and 22 h in rats given [¹⁴C-PA]- and [¹⁴C-POP]famoxadone, respectively, at 100 mg/kg bw (there was no significant difference between males and females). At a dose of 5 mg/kg bw, the plasma half-life of [¹⁴C-PA] residues was about 10.5 h. There were no significant differences in the overall fate of famoxadone in male and female rats.

The kinetic data described in the original report (Savides et al., 1995) were re-analysed Himmelstein (1999a). The justification offered for this re-analysis was that compartmental (model-dependent) analysis had been used, whereas a non-compartmental analysis would give a more accurate interpretation of terminal elimination. In so far as the calculation of the terminal elimination half-life, $T_{1/2}$, is concerned, the difference between these methods is that the non-compartmental method uses the terminal elimination constant, K_E , ($= -2.3$ slope, calculated by plotting the log of the blood concentration over linear time and calculating the slope of the terminal linear portion of the time, limited to 12–72 h in this case) in the equation $T_{1/2} = 0.693/K_E$. The compartmental method, on the other hand, is more strongly influenced by the earlier time-points on the plasma concentration–time curve for deriving an elimination rate constant. This had the effect of overestimating the $T_{1/2}$ value. $T_{1/2}$ for rats given [¹⁴C-POP]famoxadone at a dose of 100 mg/kg bw was reduced from about 22 h (compartmental method) to 15.1 ± 2.2 h and 14.5 ± 2.5 h in male and female rats, respectively (non-compartmental method). The T_{max} , C_{max} and $AUC_{(0-\infty)}$ values were not changed significantly by the re-analysis, as indicated by the ranges of means in Table 1.

The absorption, metabolism, and excretion of famoxadone was investigated in groups of five male and five female bile duct-cannulated CrI:CD BR (Sprague-Dawley) rats given a single oral dose of [¹⁴C-POP]- or [¹⁴C-PA]famoxadone at 5 mg/kg bw. Urine, bile, and faeces were collected continuously for up to 48 h after dosing. Absorption was calculated as the sum of the radioactivity in the bile, urine, cage wash, blood and carcass. The average amount of radiolabel excreted in the bile ranged from 30–39% of the administered dose. Faecal extracts contained 56–65% of the administered dose, while only 2–6% was excreted

Table 1. Ranges of mean kinetic parameters obtained using compartmental and non-compartmental analysis

Radiolabel	Dose	Parameter	Plasma		Whole blood	
			Male	Female	Male	Female
[¹⁴ C-PA]	5 mg/kg	T_{max}	2.3–3.3	3.8–4.8	4.8	6.7–8.0
		C_{max}	0.9–1.1	1.0–1.1	0.7	0.8–0.9
		$AUC_{(0-\infty)}$	18.9–19.0	20.9–21.0	29.0–29.6	44.0–46.4
[¹⁴ C-POP]	100 mg/kg	T_{max}	3.3–5.6	3.6–3.7	4.6–7.0	5.6–14.5
		C_{max}	15.4–16.4	13.4–15.6	9.9–10.3	9.4–10.9
		$AUC_{(0-\infty)}$	507–515	430–435	359–368	336–345
[¹⁴ C-PA]	100 mg/kg	T_{max}	9.5–10.0	7.0–7.5	9.5–13.9	13.3–18.0
		C_{max}	18.6–24.7	13.5–17.5	18.3–22.2	13.3–15.7
		$AUC_{(0-\infty)}$	509–511	295–296	1010–1118	1031–1082

From Savides et al. (1995) and Himmelstein (1999a)
 T_{max} , time to maximal concentration
 C_{max} , maximal concentration
 $AUC_{(0-\infty)}$, area under the concentration–time curve

in urine. At the end of the experiment (48 h), only 0.4–3.0% of the administered dose remained in the carcass. The proportion of the administered dose that was absorbed (as indicated by the amount of radiolabel found in bile, urine, blood, carcass and case-wash) was: in males and females treated with [^{14}C -PA]famoxadone, 38% and 37% respectively; in males and females treated with [^{14}C -POP]famoxadone, 41% and 37% respectively. These values were not statistically significantly different from each other. There was no difference between males or females in terms of absorption, elimination in the bile, or excretion (Savides et al., 1997).

Mice

A study was undertaken to evaluate the absorption kinetics of famoxadone in groups of 40 male Crl:CD®-1(ICR)BR mice. The time course for total radioactive equivalents in plasma was evaluated in mice given a single oral dose of [^{14}C -POP]-labelled famoxadone (radiochemical purity, 99.4%) at 50 mg/kg bw. The plasma values for T_{\max} , C_{\max} and area under the curve (AUC) were calculated for mice fed diets containing unlabelled famoxadone at a concentration of 50, 700, 2000, 3500, or 7000 mg/kg for 14 days, followed by a single oral dose of [^{14}C -POP]famoxadone. Based on the daily dietary intakes on days 7–11, these dietary concentrations provided actual doses of famoxadone of 7, 142, 367, 804, and 1500 mg/kg bw per day, respectively. At each of 10 time-points (0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after dosing), four mice per dose were killed and their blood was collected. No unchanged famoxadone was found in plasma after 14 days of dosing (limits of detection, about 0.5 $\mu\text{g}/\text{ml}$ of plasma). In general, maximum concentrations of radiolabel in plasma occurred between 1 and 4 h after dosing. The C_{\max} values for each dose were 1.6, 55, 103, 398 and 406 μg of ^{14}C -equivalents/g of plasma, respectively. The corresponding AUC values were 27, 478, 875, 2176 and 2486 μg of ^{14}C -equivalents/g of plasma per h. Thus, for doses of up to 804 mg/kg bw, the kinetic parameters increased proportionally with dose, but there were no statistically significant differences in C_{\max} and AUC between animals at 804 or at 1500 mg/kg bw. It was concluded that at dietary concentrations providing famoxadone at a dose of more than about 800 mg/kg bw, absorption from the gastrointestinal tract becomes the limiting factor for internal exposure (Himmelstein, 1999b).

Dogs

The absorption, distribution, and excretion of [^{14}C -PA]-labelled famoxadone (radiochemical purity, 97.4%) were studied in two groups of three male beagle dogs, each of which received a single oral dose at 15 mg/kg bw. One additional dog was killed four days after receiving carrier solution only. In the first group, urine, faeces, and blood were collected at specified time-points, and the dogs were killed 4 days after dosing. Dogs in the second group were killed at the time that the maximum concentration of radioactivity in plasma was observed in the first group (2 h after dosing). Erythrocytes, plasma, liver, fat, one whole eye, and the aqueous humour and the remainder of the other eye from each of these dogs were analysed for radioactivity.

In the first group, the highest mean concentration of radiolabel derived from [^{14}C -PA]famoxadone and its residues in plasma (C_{\max}) was 1.5 μg equivalents/g at a T_{\max} of 2 h. The highest mean concentration of radiolabel in erythrocytes was 0.626 μg equivalents/g at 4 h after dosing. The range for terminal half-lives ($T_{1/2}$) was 67–75 h in plasma and 146–159 h in erythrocytes. The area under the concentration–time curve ($\text{AUC}_{0-\infty}$) ranged from 96–109 $\mu\text{g}/\text{g}$ per h in plasma and from 125–135 $\mu\text{g}/\text{g}$ per h in erythrocytes. The rate of elimination was relatively slow from plasma and tissues: approximately 65% of the adminis-

tered dose was eliminated in faeces and urine within 24 h, but high concentrations of radio-label remained both in plasma and erythrocytes at 96 h after dosing. The kinetic profile for one of the three dogs in the first group was somewhat different from those obtained for the other two dogs in this group. During the first 12 h after dosing, concentrations of radioactivity in plasma and erythrocytes for this dog were similar to those for the other two dogs, but from 18 h onwards concentrations of radioactivity in plasma and erythrocytes for this dog were more than three times higher than those observed in its companions. Speculative reasons for the different kinetics in this dog were offered, such as ingestion of some of its own faeces and aspiration of its own vomit (with subsequent increased absorption from the lungs). Neither suggestion appears likely, since the concentrations of radioactivity were actually higher in plasma in the period 18–48 h and in erythrocytes in the period 18–96 h. Furthermore, no cage-side observations were recorded that would support either of these suggestions.

Urine, faeces, cage washes, and cage wipes accounted for 78.8% (range, 65.8–86.0%) of the administered dose. Of the administered dose, 70.3% was recovered from the faeces and 7.67% was found in the urine. The overall recovery of radioactivity from tissues was 0.45% of the administered dose, with a range of 0.23–0.86%. The highest concentration of radioactive residues was found in the liver (equivalents, 1.34 µg/g), followed by mesenteric fat (equivalents, 0.945 µg/g).

In the second group of dogs, killed 2 h after receiving a single dose of 15 mg/kg bw, the highest mean concentrations of residue were also found in liver (equivalents, 4.45 µg/g) and mesenteric fat (equivalents, 2.80 µg/g). The concentrations in plasma and erythrocytes were 0.999 and 0.413 µg equivalents/g, respectively. Two h after dosing, residues in the whole eye, aqueous humour and the remainder of the eye averaged 0.106, 0.061 and 0.131 µg equivalents/g, respectively (Thalacker, 1996).

1.2 Biotransformation

The CAS names and structures for the metabolites (referred to below by IN numbers) are given in the Appendix of this monograph. The proposed metabolic pathway in rats is shown in Figure 2.

In groups of five male and five female CrI:CD BR (Sprague-Dawley) rats given either [¹⁴C-POP]famoxadone or [¹⁴C-PA]famoxadone (radiochemical purities, >99% and >98%, respectively) by oral administration, unmetabolized famoxadone was the major component recovered from the faeces. The isomeric ratio of recovered parent was similar to that in the dosing material (R:S ratio, approximately 1) indicating that there was no stereoselective metabolism of enantiomers in the rat.

The primary metabolic pathway involved the hydroxylation of the intact parent molecule to the corresponding mono- and dihydroxylated derivatives (these being, respectively, IN-KZ007 and IN-KZ534), which were only recovered from the faeces. Metabolites resulting from the cleavage of the oxazolidinedione ring moiety were recovered from the urine. IN-KZ000 sulfate (metabolite M3) was the major urinary metabolite containing the [¹⁴C-POP] moiety, while 4-acetoxylaniline (IN-BY759) was the major urinary metabolite containing the [¹⁴C-PA] moiety. No parent famoxadone was detected in the urine. Several minor urinary metabolites were also observed. They were identified as the hydrolysis product of famoxadone (IN-JL856) and 4-phenoxyacetophenone (IN-H3310) from the

[^{14}C -POP] moiety. There were no significant quantitative differences in the chemical nature of the metabolites according to sex and/or treatment.

In a comparison of rats receiving a single dose of [^{14}C -PA]famoxadone at 5 mg/kg bw with rats receiving repeated doses of [^{14}C -PA]famoxadone at 5 mg/kg bw per day for 14 days, recovery of the administered dose from the urine and faeces was essentially the same, ranging from 10.4% to 11.2% in urine and from 85.7% to 89.3% in faeces. The major urinary metabolite was 4-acetoxylaniline, which constituted 4.9–8.3% of the administered dose. In faeces, unmetabolized famoxadone accounted for approximately 51–59% of the administered dose. Other metabolites identified in faeces were IN-KZ007 (males: 10.3% and 7.4% of the single and multiple doses administered, respectively; females: 13.0% and 2.8% of the single and multiple doses administered, respectively) and IN-KZ534 (males: 10.7% and 10.0% of the single and multiple doses administered, respectively; females: 7.7% and 13.4% of the single and multiple doses administered, respectively). The higher proportions of IN-KZ534 in females receiving multiple doses than in females receiving a single dose suggests self-induction of oxidative metabolism of the phenylamino moiety.

In rats receiving a single dose of [^{14}C -PA]famoxadone at either 5 or 100 mg/kg bw, the same metabolites were recovered, but it was clear that a greater proportion of the administered dose was eliminated in faeces and a smaller proportion was eliminated in urine. Recovery of the administered dose in the urine was: males, 11.2% and 4.7% at the lower and higher dose, respectively; females, 10.5% and 3.4% at the lower and higher dose, respectively. The corresponding recoveries for 4-acetoxylaniline were: males, 7.1% and 3.4%; females, 4.9% and 1.9%. Recovery of the administered dose in the faeces was: males, 87.5% and 94.6% at the lower and higher dose, respectively; females, 87.8% and 88.4% at the lower and higher dose, respectively. Of the other metabolites in faeces, IN-KZ007 in males constituted 10.3% and 2.7% of the administered dose at the lower and higher dose, respectively, and, in females constituted 13.0% and 4.3% at the lower and higher dose, respectively, and IN-KZ534 in males constituted 10.7% and 3.1% of the administered dose at the lower and higher dose, respectively, and, in females constituted 7.7% and 1.6% at the lower and higher dose, respectively (Savides et al., 1995; Savides et al., 1996).

The biliary excretion and metabolism of both the phenoxyphenyl and phenylamino radiolabels of famoxadone were examined in Crl:CD BR (Sprague-Dawley) rats. Faecal and biliary extracts were examined, but the urine was not analysed owing to the small quantities of the administered dose present (2–6%). Unmetabolized famoxadone was the only radiolabelled component detected in the faeces. The major biliary metabolites were conjugates of IN-KZ007 and catechol (IN-03492) in rats treated with [^{14}C -PA]-labelled test material, and conjugates of KZ007 and IN-ML436 in rats treated with [^{14}C -POP]-labelled test material. Parent famoxadone was not detected in the bile samples. Metabolism of famoxadone occurred via the hydroxylation of the phenoxyphenyl and phenylamino rings, hydrolysis of the oxazolidinedione moiety, cleavage of the phenylamino ring, and combinations of these pathways. Further conjugation of primary metabolites also occurred (Savides et al., 1997).

The nature of the radiolabelled metabolites present in samples of erythrocytes, plasma, liver, fat, and aqueous humour was assessed in male beagle dogs 2 h after administration of [^{14}C -PA]-labelled famoxadone as a single oral dose at 15 mg/kg bw. In addition, faeces and urine were examined for metabolites at intervals over the 96 h after dosing, and four plasma

components (famoxadone, IN-KZ007, IN-JL856, and IN-ML815) were quantified at intervals up to the 96 h time-point.

The high-performance liquid chromatography (HPLC) profiles for extracts of plasma and erythrocytes contained 11 and 12 regions of radioactivity, respectively. The identified components in these profiles of both plasma and erythrocytes were famoxadone and its metabolites IN-KZ007, IN-JL856, and IN-ML815. The major identified component in plasma was IN-KZ007, the hydroxylated derivative of famoxadone. The concentration of famoxadone was lower in plasma than in erythrocytes, suggesting that the partitioning equilibrium favoured distribution into the erythrocytes. The analysed materials that were quantified accounted for only a small proportion of the total radioactivity. The additional metabolites in plasma could not be identified.

Extracts of liver and fat were shown to contain predominantly parent compound, with lesser amounts of IN-KZ007. No further metabolites were noted in fat, but liver contained several other unidentified components. It was not possible to extract radioactivity from the aqueous humour owing to very low concentrations and small sample sizes.

A complex pattern of radiolabelled metabolites was observed in the urine and faeces. Up to eight radioactive regions were assigned to each HPLC profile for urine samples, none of which corresponded to famoxadone. These were mainly polar components, none of which corresponded to famoxadone or could be identified with reference to known metabolites of the phenylamino moiety of famoxadone. Enzyme hydrolysis provided no evidence for the presence of either glucuronide or sulfate conjugates. Faecal extracts contained primarily famoxadone at early collection times, but at later times more metabolites were formed, including IN-ML815, IN-KZ007, IN-KZ532, IN-KZ534, and IN-JL856.

Plasma samples collected at intervals up to 96 h from three dogs that had received single oral doses of [^{14}C -PA]famoxadone were analysed for famoxadone, KZ007, JL856 and ML815, using validated methods. Bimodal absorption profiles were observed for radioactivity and this was very pronounced in one dog. The metabolite KZ007 was present in the highest concentrations and its absorption profile showed a similar bimodal form. In addition, some components showed some degree of recycling, leading to multi-modal concentration/time profiles, which precluded pharmacokinetic analysis (Harrison, 1998).

1.3 Effects on enzymes and other biochemical parameters

An assessment was made of the potential of famoxadone (purity, 97.28%) to alter hepatic cytochrome (CYP) P450 content in Crl:CD[®](SD)IGS BR rats and Crl:CD-1[®](ICR)BR mice after 2 weeks of dietary exposure. Groups of five male and five female rats were given famoxadone at a dietary concentration of 0 or 20000 mg/kg, and groups of five male and five female mice were given famoxadone at a dietary concentration of 0 or 7000 mg/kg. Mean daily intakes of famoxadone over the two weeks were 1540 and 1543 mg/kgbw per day for male and female rats, respectively. For mice, the mean daily intakes were 1559 and 1633 mg/kgbw per day in males and females, respectively. After approximately 2 weeks, the rats and mice were killed, their livers were weighed, and hepatic microsomes were prepared for evaluation of total P450 content and quantification of isozymes CYP1A1, CYP2B1/2, CYP3A, and CYP4A.

In both male and female rats, treatment with famoxadone caused reductions in body-weight gain. These decrements were associated with decreased food consumption and food

use efficiency. Famoxadone caused a decrease in absolute and relative liver weights in male rats (means of 63% and 85% of the mean values for controls, respectively) and an increase in relative liver weights in female rats (a mean of 166% of the mean value for controls). The total concentration of cytochrome P450 in the livers was increased in male and female rats to 138% and 174% of the control values, respectively. There were also changes in the concentrations of specific isozymes. In male rats, concentrations of CYP2B1/2, CYP3A, and CYP4A were increased to 2452%, 228%, and 142% of control values, respectively. In female rats, concentrations of CYP2B1/2, CYP3A, and CYP4A were increased to 2759%, 363%, and 208% of control values, respectively. No alterations were observed in concentration of CYP1A1 in either male or female rats.

In mice, treatment with famoxadone did not result in alterations in body-weight gain in either sex. There were, however, increases in absolute and relative liver weights in male mice (155% and 161% of the control values, respectively) and in female mice (174% and 167% of the control values, respectively). Total hepatic concentration of cytochrome P450 was significantly increased by treatment with famoxadone (211% and 260% of the control values in male and female mice, respectively). Specific cytochrome P450 isozymes were also affected by treatment with famoxadone. In male mice, concentrations of CYP2B1/2 and CYP4A were increased to 1379% and 254% of the control values, respectively. In female mice, concentrations of CYP2B1/2 and CYP4A were increased to 940% and 401% of the control values, respectively. No alterations were observed in concentrations of CYP1A1 or CYP3A in either male or female mice (O'Connor, 1999).

These effects are attributed to a pharmacological response of the liver to exposure to a xenobiotic, which causes an induction of smooth endoplasmic reticulum and its associated enzymes. For this reason, the alterations in relative liver weight and cytochrome P450 content were considered to be adaptive responses and not adverse toxicological responses. The effects on hepatic cytochrome P450 concentrations and relative liver weight are consistent with the hepatocellular hypertrophy observed in long-term studies of toxicity in rats and mice (MacKenzie, 1996c, 1996d, 2002).

2. Toxicological studies

2.1 Acute toxicity

In an evaluation of acute oral toxicity, famoxadone (purity, 97.4%) in a suspension containing corn oil and acetone (85:15) was administered by gavage to fasted five male and five female CrI:CD-1®(ICR)BR mice (Finlay, 1994a) and five male and five female CrI:CD®BR rats (Sarver, 1994a) at a dose of 5000 mg/kg bw. The animals were observed for 14 days after dosing. There were no deaths or clinical signs of toxicity in either species and there were no statistically significant reductions in body-weight gain. No gross pathological findings were observed at autopsy. The oral median lethal dose (LD₅₀) was >5000 mg/kg bw in male and female rats and mice (Finlay, 1994a; Sarver, 1994a).

Acute percutaneous (dermal) toxicity was studied in five male and five female New Zealand white rabbits. Famoxadone (purity, 97.4%) was mixed with approximately 0.5 ml of deionized water and applied to an area of approximately 190 cm² (equivalent to approximately 10% of the total body surface) of the shaved intact skin at a dose of 2000 mg/kg bw. The application site was covered with an occlusive bandage for 24 h, then washed with soap and water. Observations for mortality and clinical signs were made approximately 3 h after dosing and then once daily for 14 days. Body weights were measured on days 1, 7 and

14 after treatment. Autopsies were performed on all rabbits after 14 days. No deaths occurred in this study. Body-weight losses of up to 6% of the initial values were observed in some rabbits 1 day after dosing. Two rabbits showed weight losses (up to approximately 2% of the previous body weight) on day 14. Slight to mild erythema was noted in four male and four female rabbits 1 day after application. On day 2, slight erythema was still observed in six rabbits. All dermal irritation had cleared by day 6 after exposure. No oedema was observed in any animal during this study. The dermal LD₅₀ for famoxadone was >2000 mg/kg bw in both male and female rabbits (Sarver, 1994b).

A study of acute toxicity after administration by inhalation was conducted to determine the median lethal concentration (LC₅₀) of famoxadone (purity, 96.1%) in five male and five female CrI:CD®BR rats. The test material was a milled particulate with a volume mean diameter of 2.5 µm suspended in air at a concentration of 5.3 mg/l air. Before the start of the study, samples of air were taken from several locations inside the exposure chamber. No statistically significant differences were observed, thereby indicating that homogeneous test atmospheres were being generated. During the 4 h exposure, the facial fur of the rats was coated with the test substance. Upon removal of rats from the restrainers immediately after the exposure, clinical signs observed included compound-stained fur and nasal discharge. Clinical signs observed on days 1–4 of the 14-day recovery period included stained perineum, ocular discharge, diarrhoea, and hunched posture. All clinical signs had resolved by day 5. There were no deaths during the study. All rats showed moderate to severe weight loss on the day after exposure to famoxadone (losses ranged from 5% to 11% of initial body weight). On day 2, four female rats experienced further, slight weight losses (losses ranged from 1% to 2% of the body weight from the previous day). All rats gained weight by the end of the 14-day recovery period, although two male rats and all female rats had instances of transient body-weight loss on one or more days. The LC₅₀ of famoxadone was >5.3 mg/l air in both male and female rats (O'Neill, 1994).

The potential of famoxadone (purity, 97.4%) to cause acute ocular irritation was evaluated in six male young adult New Zealand white rabbits. Approximately 20 mg of a white solid milled to a fine powder was administered to one eye of each rabbit. The eyes remained unwashed after treatment and observation for effects was made 1, 24, 48 and 72 h after treatment, according to the method of Draize. Biomicroscopic examinations were also made at 24 and 48 h. Initial and final body weights were recorded. There were no deaths and no adverse clinical signs. Famoxadone produced transient ocular irritation in all six rabbits. Conjunctival redness (score of 1 or 2) and chemosis (score of 1) was observed in all treated eyes 1 h after exposure. One rabbit also had iritis (score of 1), while another had occult blood in the ocular discharge. All ocular irritation was resolved by 72 h. All animals gained weight during the study (Finlay, 1994b).

The potential of famoxadone (purity, 97.4%) to cause acute skin irritation was evaluated in four male and two female New Zealand white rabbits. Approximately 0.5 g of the test material moistened with deionized water was applied, under an occlusive dressing, to the shaved back of the rabbits. After 4 h, the dressing was removed and the skin was washed with soap and warm water. There were no deaths and no significant weight loss, or clinical signs in the treated animals. No oedema occurred. Treatment with famoxadone produced very slight erythema (score 1 or 2) in four of the six rabbits within 1 h after removal of the test substance. After 72 h, the erythema had disappeared in most rabbits. All irritation cleared by day 7 (Sarver, 1994c; Finlay, 1998).

The potential of famoxadone (purity, 97.4%) to produce delayed contact hypersensitivity in male Hartley guinea-pigs was assessed by the Magnusson-Kligman maximization test. Concentrations of famoxadone used were selected on the basis of preliminary screens for irritation. Group I (20 guinea-pigs) received famoxadone, group II (20 guinea-pigs) received the famoxadone vehicle only, group III (6 guinea-pigs) served as a positive control, and group IV (6 guinea-pigs) served as a positive control for the vehicle. On the day before intradermal induction treatments, the hair of the suprascapular area was clipped. On the first day (day 1) of the experiment, three pairs of intradermal injections (each of volume 0.05 ml) were made, one of each pair on either side of the dorsal midline. The treatments administered to each group at this stage are shown in Table 2.

On day 7, the hair was again clipped and, for groups I, II and IV, the test site was treated with 3% sodium lauryl sulfate in petrolatum. On day 8, a 45 × 20 mm patch containing the topical induction dose was applied, which was covered with occlusive wrap and held in place with tape for 48 h. After unwrapping, any residual dose was removed using gauze soaked in deionized water (groups I and II) or 50% ethanol followed by deionized water (groups III and IV). The treatments used are shown in Table 3.

After clipping of both flanks of each guinea-pig on day 21, the guinea-pigs were challenged topically on day 22. Occluded topical applications were made using three chambers (diameter, 19 mm), one of which was positioned on a flank and contained vehicle only, while the other two chambers were positioned on the opposite flank and contained high and low

Table 2. Skin sensitization assay: intradermal induction

Group	Intradermal injection no.	Treatment
I	1	FCA emulsified in deionized water (1:1)
	2	5% v/v famoxadone in white mineral oil
	3	5% famoxadone in white mineral oil emulsified in FCA (1:1)
II	1	FCA emulsified in deionized water (1:1)
	2	White mineral oil
	3	White mineral oil emulsified in FCA (1:1)
III	1	FCA emulsified in deionized water (1:1)
	2	0.1% DNCB in 50% ethanol: saline
	3	0.1% DNCB in 50% ethanol: saline emulsified in FCA (1:1)
IV	1	FCA emulsified in deionized water (1:1)
	2	50% ethanol in saline
	3	50% ethanol: saline emulsified in FCA (1:1)

From Moore (1994)

FCA, Freund complete adjuvant

DNCB, 1-chloro-2,4-dinitrobenzene

Table 3. Skin sensitization assay: topical induction

Group	Treatment
I	0.4 g famoxadone mixed with 0.4 ml white mineral oil
II	0.4 ml white mineral oil
III	0.4 ml 0.1% DNCB in 50% ethanol: saline
IV	0.4 ml 50% ethanol: saline

From Moore (1994)

DNCB, 1-chloro-2,4-dinitrobenzene

Table 4. Skin sensitization assay: challenge

Group	Application site	Treatment
I and II	Right flank	0.1 g white mineral oil
	Left flank	0.1 g famoxadone and 0.1 g white mineral oil
	Left flank	0.1 g of 33% (w/w) famoxadone in white mineral oil
III and IV	Right flank	0.1 ml acetone
	Left flank	0.1 ml 0.1% DNCB in acetone
	Left flank	0.1 ml 0.03% DNCB in acetone

From Moore (1994)

DNCB, 1-chloro-2,4-dinitrobenzene

doses of the appropriate material. The chambers were covered and strapped in place for 24 h. After removal of the chambers, the sites were wiped with the appropriate vehicle. These treatments are described in Table 4.

One guinea-pig was found dead on day 14. At autopsy, blood was found in the pericardial sac. All other guinea-pigs appeared to be normal. Skin reactions were scored at 24 and 48 h after removal of the chambers. No dermal irritation was observed at either time-point in the groups treated with famoxadone or in the vehicle control group, while all of the guinea-pigs treated with 1-chloro-2,4-dinitrobenzene (DNCB) showed moderate redness at both doses. It was concluded that famoxadone did not produce delayed contact hypersensitivity (skin sensitization) (Moore, 1994).

2.2 Short-term studies of toxicity

Mice

In a 14-day feeding study, groups of five male and five female Crl:CD-1®(ICR)BR mice received diets containing famoxadone (purity, 97.4%) at a concentration of 0, 100, 1250, 3500, or 7000 mg/kg (equal to 0, 15.7, 204, 553 or 1094 mg/kg bw, respectively, in males and 0, 18.1, 236, 647 or 1291 mg/kg bw, respectively, in female). At the end of the period of feeding, all mice were killed and autopsied. Only the liver was examined microscopically. No deaths occurred and there were no compound-related clinical signs of toxicity. There were no statistically significant effects on mean body-weight gain, food consumption, or food use efficiency in either sex. No compound-related gross lesions were observed at necropsy. There was, however, a dose-related increase in mean absolute and mean relative liver weights at all doses. Mean absolute liver weights were statistically increased at 1250, 3500, and 7000 mg/kg in males and at 3500 and 7000 mg/kg in females. The increase in mean relative liver weights were statistically significant at 1250, 3500, and 7000 mg/kg in both male and female mice. Mean absolute and relative liver weights at 100 mg/kg were greater than those of controls, but were not statistically significant. Compound-related centrilobular hepatocellular hypertrophy was noted at 1250, 3500, and 7000 mg/kg in both males and females. Slight centrilobular fatty changes occurred in some mice of both sexes at 3500 and 7000 mg/kg. Some single-cell necrosis of hepatocytes was seen in females at 3500 and 7000 mg/kg. The hypertrophy found at 1250 mg/kg was considered to be a pharmacological response to exposure to the compound, while the fatty change and single-cell necrosis observed at 3500 and 7000 mg/kg were suggestive of slight hepatotoxicity. The NOAEL in male and female mice treated with famoxadone in the diet for 14 days was 1250 mg/kg (equal to 204 and 236 mg/kg bw per day, respectively) on the basis of slight hepatotoxicity observed at 3500 mg/kg (Ghantous, 1999).

The effect of famoxadone (purity, 97.7%) on blood chemistry and hepatic biochemical parameters was evaluated in Crl:CD-1®(ICR)BR mice treated for either 14 or 28 days. In the 14-day study, groups of 10 male and 10 female mice were fed diets containing famoxadone at a concentration of 0 or 3500 mg/kg. In the 28-day study, groups of 10 male and 10 female mice were fed diets containing famoxadone at a concentration of 0, 100, 500, 1000, 2000, 2500 or 3000 mg/kg. No compound-related effects on body weight, body-weight gain, or clinical signs of toxicity were produced by dietary concentrations of 3500 mg/kg for 14 days, or ≤ 3000 mg/kg for 28 days. Absolute and relative liver weights were increased in male mice at ≥ 1000 mg/kg and in female mice at ≥ 500 mg/kg. After 14 and 28 days of treatment, increases in liver-specific serum enzymes (alkaline phosphatase, alanine aminotransferase, and sorbital dehydrogenase) were observed at ≥ 1000 mg/kg. These increases over the values of the control group were generally small (less than two-fold) and were not dose-dependent. Serum concentrations of triglyceride were not affected at any dose. Compound-related increases in hepatic β -oxidation activity were also observed in males and females at ≥ 1000 mg/kg. Total hepatic cytochrome P450 content was increased in all mice treated with famoxadone. The NOAEL in male and female mice receiving diets containing famoxadone for 28 days was 500 mg/kg on the basis of increases in the activities of serum enzymes derived from hepatic cytosol and in peroxisomal β -oxidation activity at ≥ 1000 mg/kg (MacKenzie, 1996a).

Groups of 20 male and 20 female Crl:CD-1®BR mice were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 35, 350, 3500 or 7000 mg/kg (equal to 0, 5.89, 62.4, 534 or 1149 mg/kgbw, respectively, for male mice and 0, 8.21, 79.7, 757 or 1552 mg/kgbw, respectively, for female mice) for approximately 90 days. There were no treatment-related deaths, clinical signs, or ophthalmic changes. Ophthalmology revealed only a few cases of phthisis bulbi (males: 2/10, 1/10, 0/10, 2/10 and 1/10, respectively; females: 1/10 only at 7000 mg/kg) and one complete cataract in one male at 350 mg/kg.

It was reported that there was a mild regenerative, Heinz body-associated, haemolytic anaemia in both sexes at 3500 and 7000 mg/kg and that this was characterized by decreases in erythrocyte count and increases in reticulocyte count, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. However, the variations in erythrocyte count were almost random in male mice, being significantly elevated at 35 and 350 mg/kg after both 45 and 90 days and significantly depressed at 7000 mg/kg only after 45 days. Similarly, the concentrations of haemoglobin in males did not fit a diagnosis of anaemia in males, these concentrations being significantly elevated at 3500 and 7000 mg/kg after 45 days and at 35, 350 and 7000 mg/kg after 90 days. Erythrocyte volume fractions were increased at 35 and 350 mg/kg after 45 and 90 days and at 3500 mg/kg after 45 days. On the other hand, there was clearly a treatment-related increase in circulating reticulocytes that reached significance at 3500 and 7000 mg/kg after 45 and 90 days, while mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentrations were significantly elevated at 7000 mg/kg after both 45 and 90 days. A somewhat similar confusing combination of haematological data was obtained in female mice, in which erythrocyte counts were depressed while haemoglobin concentrations were increased at 7000 mg/kg after both 45 and 90 days. Erythrocyte volume fractions were depressed at 7000 mg/kg after 45 days, but there were no significant changes after 90 days. On the other hand, there was again a clearly dose-related increase in circulating reticulocytes that reached significance at 3500 and 7000 mg/kg after 45 and 90 days, while mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentrations were significantly elevated at 3500 and 7000 mg/kg after

both 45 and 90 days. A mild leukocytosis in both sexes at 7000 mg/kg was considered to be secondary to haemolysis. Platelet counts were significantly depressed in all treated groups of males after 45 days and in females after 90 days. Increased spleen weights at 3500 (females only) and 7000 mg/kg and the microscopic finding of increased red pulp (i.e. congestion) in the spleen (females, 3500 mg/kg; males and females, 7000 mg/kg) correlated with the haematological findings. Increased haemosiderin pigment in the spleens and bile pigment in the livers of both sexes at ≥ 3500 mg/kg were also considered to be secondary to the haemolysis. Blood chemistry was not evaluated in this study.

In both male and female mice at 3500 mg/kg and 7000 mg/kg, liver weights were increased and there was centrilobular necrosis, diffuse fatty change and increased bile pigment. In addition, there were increases in total cytochrome P450 content and β -oxidation activity. Hepatic cell proliferation indices, as measured by the incorporation of 5-bromo-2'-deoxyuridine delivered by implanted osmotic pumps were increased in female, but not male mice in both of these groups. At 350 mg/kg, there was an increase in total cytochrome P450 content in females, which was considered to be a non-adverse, pharmacological response. The NOAEL in male and female mice receiving diets containing famoxadone for 90 days was 350 mg/kg, equal to 62.4 and 79.7 mg/kg bw per day, respectively, on the basis of mild haemolytic anaemia and mild hepatotoxicity at 3500 mg/kg (Biegel, 1994; Saik, 1994).

Rats

Groups of five male and five female Crl:CD®BR rats were fed diets containing famoxadone (purity, >98%) at a concentration of 0, 100, 1000, 6000, or 20 000 mg/kg for 14 days. These dietary concentrations provided doses equal to 0, 8.97, 85.8, 428 or 1629 mg/kg bw, respectively, for males and 0, 8.85, 80.9, 440 or 1589 mg/kg bw, respectively, for females. After 14 days, all rats were killed and subjected to examination post mortem. No deaths occurred during the study. Piloerection was observed in rats of both sexes at 6000 mg/kg and 20 000 mg/kg, and hyperactivity, abnormal gait or mobility was observed in a few females at the same concentrations. Mean daily food consumption was significantly decreased in males and females at 1000, 6000, and 20 000 mg/kg. In males at 1000 mg/kg, this decrease was only statistically significant during the first week and was not considered to be an adverse effect as it did not result in decreased weight gain or food use efficiency. Body weights and body-weight gains were statistically significantly decreased in males at 6000 and 20 000 mg/kg and in females at 1000, 6000, and 20 000 mg/kg. There were no compound-related gross lesions. Statistically significant increases in mean relative liver weights were seen in females at 1000, 6000, and 20 000 mg/kg and in males at 20 000 mg/kg. Microscopic compound-related lesions were observed in the livers of males and females at 1000, 6000, and 20 000 mg/kg and consisted of hepatocellular hypertrophy, hepatocellular degeneration and single-cell necrosis, and an increase in hepatocellular mitotic figures. While the hepatocellular hypertrophy and the associated increases in relative liver weights were considered to be pharmacological (i.e. non-adverse) responses, the single-cell necrosis and increase in mitotic figures represented slight hepatotoxicity. The NOAEL in male and female rats receiving diets containing famoxadone for 14 days was 100 mg/kg, equal to 8.97 and 8.85 mg/kg bw per day, respectively, on the basis of the slight hepatotoxicity observed at 1000 mg/kg (MacKenzie, 1992; Slone, 1991).

Groups of 10 male and 10 female Crl:CD®BR rats were fed diets containing famoxadone (purity, 97.7%) at a concentration of 0, 100, 200, 300, 400, 500, or 600 mg/kg, for

28 days. Food consumption was not measured in this study. These dietary concentrations were equivalent to doses of 0, 10, 20, 30, 40, 50 or 60 mg/kg bw per day. Blood samples were collected and evaluated for effects on markers of hepatotoxicity after 14 and 28 days. No compound-related effects on body-weight gain or clinical signs of toxicity were produced at any concentration. At ≥ 400 mg/kg, minimal increases in liver-specific enzymes (alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase) were observed in males, while sorbitol dehydrogenase activity was significantly increased in females. The latter enzyme provides the most sensitive indication of hepatotoxicity. In addition, decreases in concentrations of serum triglycerides were observed in females at ≥ 400 mg/kg, but only after 28 days. The results of this study suggest that administration of famoxadone in the diet causes minimal hepatocellular toxicity at dietary concentrations of ≥ 400 mg/kg in male and female rats. The NOAEL was 300 mg/kg in both male and female rats receiving diets containing famoxadone for 28 days, on the basis of increased activities of liver cytosolic enzymes released into serum, indicative of minimal hepatotoxicity at 400 mg/kg (MacKenzie, 1996b).

Groups of 20 male and 20 female CrI:CD®BR rats were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 50, 200, 800 or 1600 mg/kg, for approximately 90 days. These dietary concentrations provided doses equal to 0, 3.34, 13.0, 52.1 and 106 mg/kg bw, respectively, for males and 0, 4.24, 16.6, 65.7, and 130 mg/kg bw, respectively, for females. Cell proliferation was evaluated in the livers of five rats of each sex per group after approximately 14 days of treatment and blood samples were taken after 45 and 90 days. At the end of the 90-day period of feeding, all rats were killed and subjected to examination post mortem. There were no compound-related deaths, clinical signs, or ophthalmic effects. One male rat at 200 mg/kg was accidentally killed on day 11 and another male from the same group was found dead with septicaemia on day 42. Compound-related and biologically relevant decreases in body-weight gain were observed in females at 200 mg/kg (to 91% of the controls) and in both males and females at 800 mg/kg (to 91% and 87% of the controls, respectively) and at 1600 mg/kg (to 85% and 85% of the controls, respectively). Although the reductions in body-weight gain were not statistically significant in females at 200 mg/kg or in males at 800 mg/kg, they were considered to be biologically significant, since they represented part of a treatment-related response. There were few ophthalmic changes and these were not treatment-related. They consisted of one case of phthisis bulbi in a male at 800 mg/kg and one case of radial linear retinal atrophy in a female at 1600 mg/kg.

Mild haemolytic anaemia was observed in both sexes after both 45 and 90 days at 800 and 1600 mg/kg. The diagnosis was much clearer than was the case for mice (see above). It was characterized by decreases in erythrocyte counts and haemoglobin in males and females after both 45 and 90 days at 200, 800 and 1600 mg/kg, and in erythrocyte volume fractions in males and females at after both 45 and 90 days 800 and 1600 mg/kg, as well as in females after 90 days at 200 mg/kg. There were clearly dose-related increases in reticulocyte counts that reached statistical significance in males and females after both 45 and 90 days at 800 and 1600 mg/kg, while mean corpuscular volume and mean corpuscular haemoglobin were increased in males and females after both 45 and 90 days at 800 and 1600 mg/kg, and mean corpuscular volume was additionally increased in females at 200 mg/kg at both of these sampling times. Mean corpuscular haemoglobin concentrations were unaltered in either males or females at any dose or sampling time. On the basis of the reticulocytosis, the anaemia was described as regenerative. Decreases in erythrocyte counts and haemoglobin in male and female rats at 200 mg/kg and erythrocyte volume fractions in

females at 200 mg/kg were not accompanied by reticulocytosis. The authors stated that the changes observed at 200 mg/kg were not biologically important because they were not accompanied by reticulocytosis. It is noted, however, that the reticulocyte counts were higher in both males and females at 200 mg/kg than at 0 and 50 mg/kg. Increased spleen weights and microscopic findings in the spleen (haemosiderin deposition, extramedullary haematopoiesis, and congestion) and bone marrow (hyperplasia) correlated with the haematological findings.

Hepatotoxicity occurred in both sexes at 800 and 1600 mg/kg, but it was more severe in males, being characterized by statistically significant, increased serum concentrations of the liver enzymes alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase. Concentrations of bilirubin were also increased in males in these two groups. In females, only concentrations of the most sensitive marker of hepatotoxicity, sorbitol dehydrogenase, were increased. These effects were considered to be evidence of hepatocellular injury or necrosis and cholestasis. Scattered white foci in the livers were found during gross examination at autopsy in males at 1600 mg/kg, which correlated with the prominent microscopic focal degeneration found in this group. Mean absolute liver weights were decreased in the males at 800 mg/kg and 1600 mg/kg, while they were increased in the females at these dietary concentrations. Liver weights relative to body weights were significantly increased in females receiving dietary concentrations of ≥ 200 mg/kg. In addition to the focal degeneration mentioned above, other liver pathology observed in males and females at 800 mg/kg and 1600 mg/kg were centrilobular hypertrophy, single-cell necrosis (apoptosis), an associated increase in mitotic figures and bile duct hyperplasia. Cell proliferation tests showed that the 5-bromo-2'-deoxyuridine labelling indices in the groups receiving famoxadone at dietary concentrations of 800 and 1600 mg/kg was approximately 18–19 times higher than the value for male controls and approximately two-fold and five-fold higher than the control value in these two groups, respectively, in females. The incidence and severity of these lesions was greatest in males at 1600 mg/kg. Centrilobular hypertrophy, the most common lesion found, often results from hypertrophy of the smooth endoplasmic reticulum and increases in the enzymes associated with this organelle, and may be associated with proliferation of peroxisomes. While biochemical tests showed no treatment-related increase in total cytochrome P450 in the liver, hepatic β -oxidation was increased approximately two-fold in males and females at 800 mg/kg and 1600 mg/kg and approximately 1.5-fold in females at 200 mg/kg, these increases probably representing an adaptive response.

Other blood chemistry findings were considered to be secondary to hepatotoxicity rather than direct effects of treatment. These included increased serum concentrations of cholesterol in females, slightly lowered serum concentrations of glucose and globulin in males and females and decreased concentrations of total protein in males. The increased cholesterol in females may have been due to alterations in lipid metabolism, rather than hepatotoxicity, since a similar effect was not observed in males in spite of evidence of more severe hepatotoxicity in this sex. Increased mean liver weights in females at 800 and 1600 mg/kg were attributed to hepatocellular hypertrophy, while decreased liver weights in males at 800 and 1600 mg/kg were attributed to the more severe hepatotoxicity observed in this sex.

The NOAEL in male and female rats receiving diets containing famoxadone for 90 days was 200 mg/kg and 50 mg/kg, respectively, equal to 13.0 and 4.24 mg/kg bw per day, respectively, on the basis of hepatotoxicity and haemolytic anaemia at 800 mg/kg, equal to

52.1 mg/kgbw in males (and to 65.7 mg/kgbw in females) and decreased body-weight gain at 200 mg/kg, equal to 16.6 mg/kgbw in females (MacKenzie, 1995; Sykes, 1995; MacKenzie, 1999). It is noted, however, that the reduction in body-weight gain in female rats at 200 mg/kg is not supported by observations made in three other studies of comparable or longer duration in rats. These are the 24-month study of toxicity/carcinogenicity, a two-generation study of reproduction and a 3-month study of neurotoxicity. The same strain of rat was used in all four studies. Consequently, the NOAEL identified on the basis of body-weight gain reduction from this 3-month study toxicity was not used in the evaluation made by the Meeting.

Groups of 10 male and 10 female CrI:CD (SD)®IGS BR rats received daily applications of famoxadone (purity, 97.3%) to the shaved, intact skin at a dose of 0, 250, 500, or 1000 mg/kgbw per day for approximately 28 days. The test site was covered with gauze dressing under layers of stretch gauze and self-adhesive bandages. The period of exposure was approximately 6 h/day. After treatment, the bandages were removed, and excess test substance was washed from the skin using water and mild soap. Body weight, food consumption, and clinical signs were evaluated throughout the study. Blood samples were collected before the rats were killed and subjected to examination post mortem.

There were no test substance-related effects on mortality, clinical signs of toxicity, body-weight gain or food consumption. Females in the groups receiving a dose of 500 or 1000 mg/kgbw per day had slight decreases in erythrocyte counts and haemoglobin concentration, but in the absence of other haematological effects, these small changes were not considered to be biologically significant. Clinical chemistry parameters were unaffected by treatment with famoxadone. Statistically significant increases in alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase activities were present at 500 and 1000 mg/kgbw per day and were considered to be indicative of minimal hepatocellular toxicity. These effects were not observed in females.

Liver weights were increased in all treated male and female groups. Relative to body weight, the liver weight increases at 250, 500 and 1000 mg/kgbw were, in male rats, 10%, 12% and 21%, respectively, and, in female rats, 11%, 14% and 9%, respectively. These increases were statistically significant in males at 1000 mg/kgbw per day in absolute and relative (to body and to brain weights) terms, and in males at 500 mg/kgbw per day group in relative (to body weight) terms. At 500 and 1000 mg/kgbw per day, the principal microscopic change observed in males and females was minimal hypertrophy of centrilobular hepatocytes, which may have been responsible for the weight changes. In the absence of more definitive microscopic evidence of hepatocellular toxicity, these changes were judged not to be adverse. In males at 500 and 1000 mg/kgbw per day, hypertrophy was associated with low incidences of apoptosis in the liver. Apoptosis has been reported to occur after the administration of substances that induce liver enzymes, perhaps as a homeostatic mechanism to eliminate excess cells, rather than as a result of primary cytotoxicity (Bursch et al., 1985). Additionally, incidences of minimal focal necrosis of the liver were increased in females receiving the highest dose (0/10, 0/10, 1/10 and 4/10 at 0, 250, 500 and 1000 mg/kgbw per day, respectively), but not in males (1/10, 0/10, 2/10 and 2/10 at 0, 250, 500 and 1000 mg/kgbw per day, respectively). The fact that male rats appear to be more sensitive than female rats to the hepatotoxic effects of famoxadone administered orally would suggest that the slightly higher incidence of focal necrosis observed in females at the highest dose in this study of dermal administration is not related to treatment.

In the treated males, increased incidences of extramedullary haematopoiesis were observed in the spleen, but neither the incidences nor the severity of the effect were dose-related and there were no associated haematological effects in males (the reductions in erythrocyte counts and haemoglobin, mentioned above, were in female rats).

The NOAEL for famoxadone administered dermally was 250mg/kgbw per day in male rats, on the basis of slight increases in liver enzymes, which were suggestive of minimal hepatotoxicity. The NOAEL in female rats was 1000mg/kgbw per day, the highest dose tested (Ladics, 1998)

Dogs

In a study designed to assess palatability and toxic potential, groups of two male and two female outbred beagle dogs were fed diets containing famoxadone (purity, 97.4%) for 5 weeks. An initial dietary concentration of 250mg/kg was selected, which was increased to 500mg/kg after the first week of dosing, to 1000mg/kg after week 2, and to 2000mg/kg after week 3. Famoxadone was administered at a dietary concentration of 2000mg/kg for the remainder of the study. A concurrent control group was included in the study. Survival was unaffected by the administration of famoxadone. Transitory myotonic twitches in all four treated dogs were observed on the last 1–2 days of dosing at 2000mg/kg. Slight decreases in body-weight gain and food consumption were noted in males and/or females receiving a dietary concentration of 500mg/kg, which became marked in both males and females when the dietary concentration was increased to 1000 and 2000mg/kg. Gross examination post mortem and measurements for liver enzyme activity in the serum revealed no effects of treatment (Tompkins, 1994).

Groups of four male and four female outbred beagle dogs were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 40, 300, or 1000mg/kg (the last concentration was reduced to 600mg/kg during week 4), for 90 days. These dietary concentrations provided doses equal to 0, 1.3, 10.0 and 23.8 (reduced to 21.2) mg/kgbw, respectively, for males and 0, 1.4, 10.1 and 23.3 (reduced to 20.1) mg/kgbw, respectively, for females. The reduction in dose was made because of the occurrence of myotonic twitches in both sexes and convulsions in one female. These twitches, which usually began approximately 4h after feeding, and were first observed in all males and females in week 3, continued until the end of the study. The only other treatment-related clinical sign was soft stools in males and females at the highest dose. There were no deaths during the period of treatment. Mean body weights in the groups treated with 1000–600mg/kg were lower than those of the controls by 11% in males and 14% in females after 13 weeks. These reductions were attributable to the effects of the higher dose experienced during the first 4 weeks.

A mild regenerative haemolytic anaemia was observed in both sexes at 1000–600mg/kg. The effect was greater at week 5 (1000mg/kg) than it was at week 12 (1000–600mg/kg), as would be expected following a reduction in the dietary concentration (although the reduction in dose appears to have been quite small). This was characterized by decreases in erythrocyte counts, erythrocyte volume fraction, haemoglobin and mean corpuscular haemoglobin concentration. At the same time there were increases in reticulocyte count, Heinz body count, mean corpuscular volume, mean corpuscular haemoglobin concentration, and mean platelet count. A few statistically significant changes in haematological parameters were observed in females at 40mg/kg and in males and females at 300mg/kg, which comprised decreases in erythrocytes, erythrocyte volume fraction

and haemoglobin. However, since reticulocyte counts were not elevated and most erythrocyte values were within the ranges for historical controls and/or were similar to the values measured before treatment began, the effect was not considered to be toxicologically significant at 40 and 300 mg/kg. Increased haemosiderin deposition in the liver of males at 300 mg/kg and males and females at 1000–600 mg/kg and in the bone marrow of females at 300 mg/kg and males and females at 1000–600 mg/kg was probably a consequence of the haemolytic anaemia.

The only treatment-related effect on blood chemistry parameters was a statistically significant increase in mean potassium concentrations at week 5 (fasted and non-fasted) and/or week 12. It was suggested that these elevations in potassium concentration were responsible for the myotonic twitching seen in the group receiving the highest dose. However, while the twitching persisted through the study, the concentrations of potassium at week 12 were no different to those for males in the control group (4.59 ± 0.163 versus 4.77 ± 0.227 milliequivalents per litre (meq/l)), although they did remain statistically significantly elevated in females.

Hepatotoxicity was not observed, and the activities of liver enzymes in serum, mean liver weights, and liver histology were not affected by administration of famoxadone in the diet.

Bilateral posterior cortical lens opacities—cataracts—(graded as slight) were observed at week 12 in males (2/4) and females (1/4) at 300 mg/kg and in males (2/4) and females (2/4) at 1000–600 mg/kg. None of the animals showed signs of visual impairment. Microscopically, minimal to mild treatment-related lenticular degeneration was seen in males (4/4) and females (4/4) at 300 mg/kg and males (3/4) and females (4/4) at 1000–600 mg/kg. One female in the group receiving famoxadone at a dietary concentration of 40 mg/kg had a unilateral lens lesion that was graded as minimal. The lenticular degeneration was characterized by a small focal zone of swollen lens fibers present at the Y suture of the posterior lens capsule. In some animals, these fibres formed morgagnian corpuscles, a change commonly associated with clinical lens opacities.

In this 90-day feeding study in dogs, the NOAEL for famoxadone in males was 40 mg/kg, equal to 1.3 mg/kg bw per day, and undetermined in females, on the basis of clinical and microscopic evidence of slight posterior subcortical lenticular opacities in males and females at 300 mg/kg, equal to 10.0 and 10.1 mg/kg bw per day, respectively. No NOAEL was identified for females because there was minimal microscopic unilateral lens degeneration in one out of four dogs at 40 mg/kg, equal to 1.4 mg/kg bw per day, the lowest dose tested (Saik, 1995; Tompkins, 1995).

Groups of four male and four female outbred beagle dogs were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 10, 20, 40, 300, or 300 mg/kg (the second high dose group being used as a recovery group, receiving famoxadone for the first 3 months and basal diet for the remaining 9 months), for 1 year. These dietary concentrations provided doses equal to 0, 0.3, 0.6, 1.2, 8.8 and 10.1 mg/kg bw per day, respectively, for males and 0, 0.3, 0.6, 1.2, 9.3 and 9.9 mg/kg bw per day, respectively, for females. There were no deaths during the study and there were no treatment-related effects on body weights, clinical signs of toxicity, organ weights, haematology, blood chemistry, urine analysis, or gross pathology. Microscopically, no evidence of hepatotoxicity was observed.

Treatment-related effects were limited to the lenses (i.e. occurrence of cataracts) of males and females at 300mg/kg, and at 300mg/kg followed by a recovery period. Ophthalmological examinations were conducted during study weeks -1 and 2, 8, 12, 16, 20, 25, 40 and 50. Posterior subcapsular lens opacities were observed in 2/4 males and 2/4 females at 300mg/kg, and in 4/4 males and 4/4 females in the group receiving 300mg/kg followed by a recovery period. Most of the lesions first appeared between weeks 8 and 12. The extent and progression of these lesions were variable. In no dog did the entire lens become opaque and no dog became clinically blind during the study. Regression of the lesion did not occur in any dog exposed to famoxadone for the entire year, however, regression was noted in some dogs in the group receiving 300mg/kg followed by a recovery period. Clinical resolution of small opacities was noted in one eye of each of two dogs in the group receiving 300mg/kg followed by a recovery period, although most of the posterior capsular opacities did not completely regress. During the period that dogs in the group receiving 300mg/kg followed by a recovery period were receiving the control diet, no new ocular lesions developed, and most of the existing ocular lesions did not progress in severity. During this period, two dogs developed prolapsed third eyelids and one female showed progression of anterior Y lens opacities. Equatorial lens opacities, occasionally extending into the cortical regions of the lens, were observed in two out of four males and two out of four females at 300mg/kg. These lesions developed after 6–12 months of exposure to the test substance and were not dependent on the previous development of a posterior subcapsular lesion. No other treatment-related ocular changes were observed. Intraocular pressure, measured at 3 months, did not differ among test groups.

Microscopic examination of the eyes demonstrated treatment-related lenticular degeneration in males and females at 300mg/kg and in the group receiving 300mg/kg followed by a recovery period. These lesions were characterized by fibre swelling with formation of morgagnian corpuscles and clefts within the lens cortex. Lenticular degeneration (including posterior subcapsular and equatorial degeneration) was observed in three out of four males and two out of four females at 300mg/kg, and in two out of four males, and four out of four females in the group receiving 300mg/kg followed by a recovery period. Microscopic findings in the lenses of dogs at 300mg/kg and at 300mg/kg followed by recovery were highly correlated, both in incidence and location, with the results of clinical ophthalmology.

In this 1-year feeding study in dogs, the NOAEL for famoxadone was 40mg/kg in males and females, equivalent to 1.2mg/kgbw per day in both sexes, on the basis of clinical and microscopic evidence of ocular lesions in both sexes at 300mg/kg, equivalent to 8.8 and 9.3mg/kgbw per day in males and females, respectively (Mertens, 1996; Frame, 1998).

Monkeys

Groups of four male and four female cynomolgus monkeys were given famoxadone (purity, 97.4%) orally by gavage at a dose of 0, 1, 100, or 1000mg/kgbw per day for 52 weeks. The monkeys were observed for signs of toxicity, body-weight changes, and food consumption. Ophthalmic examinations were performed before treatment began and during weeks 5, 13, 26, 39, and 52, while haematology, blood chemistry, and urine analysis were performed before treatment began and during weeks 5, 13, 26, 39, and 53. After 53 weeks, the monkeys were killed and subjected to gross and microscopic examinations and organ weight analysis. Four pathologists examined the eyes of all of the animals.

Two monkeys died during the study (one male on day 14 at 1 mg/kg bw per day and one female on day 77 at 1000 mg/kg bw per day). It was concluded by the examining veterinarian that the causes of death were not treatment-related. Body weight, body-weight gain, and food consumption were unaffected by treatment. During the course of the study, there was the intermittent occurrence of white faeces in some monkeys at 1000 mg/kg bw per day, which was not considered adverse. The only adverse treatment-related effect observed was a mild haemolytic anaemia in both sexes at 1000 mg/kg bw per day. A slightly lower erythrocyte count, haemoglobin concentration, and erythrocyte volume fraction with secondary microscopic changes in the spleen, liver and kidney were observed in males and females at this dose. Blood chemistry parameters, organ weights (absolute and relative to body weight and to brain) showed no compound-related changes at any dose.

Throughout the course of the study, there was no clinical or microscopic evidence of lenticular opacities in any monkey at any dose. It is noted that the highest dose in this 1-year study (1000 mg/kg bw per day) is approximately 700-fold greater than the LOAEL (1.2 mg/kg bw per day) for ocular effects observed in the 1-year study in dogs (see above). These results indicate that primates either do not develop cataracts induced by famoxadone or that they are significantly less sensitive than dogs. The NOAEL for famoxadone was 100 mg/kg bw per day in male and female cynomolgus monkeys, on the basis of a mild haemolytic anaemia noted in both sexes at 1000 mg/kg bw per day) (Williams, 1997; Sykes, 1998).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 80 male and 80 female Crl:CD-1®(ICR)BR mice were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 5, 50, 700, or 2000 mg/kg for approximately 18 months. These dietary concentrations provided doses equal to 0, 0.70, 6.78, 95.6 or 274 mg/kg bw per day for males, respectively, and 0, 0.96, 9.84, 130 or 392 mg/kg bw per day for females, respectively. The study complied with European Commission (EC) directive 87/302/EEC and was conducted according to the data requirements of the United States Environmental Protection Agency (EPA) pesticide assessment guidelines subdivision F 83-2, OECD test guideline 451 and the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan Nohsan No. 4200. Ophthalmology was performed on all mice before treatment, after 9 months of treatment and at the end of the study. Blood samples were taken from 10 mice of each sex per group after approximately 3, 6, 12 and 18 months of treatment. Cell proliferation in the liver was measured on five mice of each sex per group after 2 weeks and 9 months and, additionally, five mice of each sex per group were killed at each of these same times for measurement of total hepatic cytochrome P450 content and the activity of peroxisomal β -oxidation enzymes.

There were no compound-related effects on survival, clinical signs, body weights, body-weight gains, or haematology. Ophthalmology performed on all surviving mice after approximately 9 months and near the end of the study revealed no treatment-related abnormalities. Observations recorded consisted mainly of a diffuse retinal degeneration that showed no dose-related response in either males or females; three cases of phthisis bulbi (one in each of three different groups of males) and focal cataracts (one male and one female at 50 mg/kg and one male and one female at 700 mg/kg). Blood chemistry was limited to plasma protein concentration measurements, which were not significantly different across the groups. Urine analysis was not performed in this study.

Mean absolute and relative (to body weight and brain weight) liver weights were observed in male and female mice at 700 and 2000 mg/kg and mean absolute liver weight was also significantly increased in female mice at 50 mg/kg. These liver weight changes were associated with mainly centrilobular hepatocellular hypertrophy in both sexes at 700 and 2000 mg/kg and with an equivocal level of this change in females at 50 mg/kg. The hypertrophy observed in these two groups was associated with increases in smooth endoplasmic reticulum and peroxisomes. Increases in total cytochrome P450 concentration to approximately 2–2.5-fold the control values were observed after 2 weeks and 9 months. Hepatic β -oxidation was significantly increased in male mice at 700 and 2000 mg/kg after 2 weeks (1.8-fold and 2.3-fold, respectively) and in male mice at 2000 mg/kg after 9 months (1.8-fold). The rates of hepatic β -oxidation were significantly increased in female mice at 2000 mg/kg after 2 weeks (2.0-fold) and in female mice at 700 and 2000 mg/kg after 9 months (1.9-fold and 2.0-fold, respectively). Microscopic lesions that were collectively considered to be indicative of liver toxicity included increases in diffuse fatty change, focal hepatocellular necrosis in males at 2000 mg/kg, eosinophilic foci in males at 700 and 2000 mg/kg, apoptosis (females), and the accumulation of haemosiderin and lipofuscin in Kupffer cells of both sexes. The biological significance of some of these observations is not clear. Thus, haemolysis was not observed, which might have explained the haemosiderin deposition and the eosinophilic foci showed no dose–response relationship and were not associated with neoplasia. Lipofuscin would be indicative of lipid oxidation. No statistically significant increases in cell proliferation in the livers of male or female mice were observed at either 2 weeks or 9 months.

An increase in the incidence of systemic amyloidosis and amyliodosis as a cause of death was observed in female mice at 2000 mg/kg. The toxicological significance of this increased incidence is unclear as it most likely is indicative of altered homeostasis, but it is considered to be evidence of treatment-related toxicity in the female mice at 2000 mg/kg.

There were no statistically significant increases in tumour incidence in the groups treated with famoxadone. Of the neoplasms observed, the only one requiring comment is malignant lymphoma, found at higher incidence in female mice at 2000 mg/kg. The incidences were 1/60, 2/60, 2/61, 0/60 and 6/60 at 0, 5, 50, 700 and 2000 mg/kg, respectively. The higher incidence was not statistically significant and was within the range for historical controls for the laboratory over a relevant period (1992–1994), when the numbers of mice with lymphomas in control groups in seven experiments were 0/80, 2/80, 9/80, 19/80, 7/80, 3/80 and 0/80. The NOAEL for famoxadone in mice after 18 months of dietary exposure was 700 mg/kg in males and females, equal to 95.6 and 130 mg/kg bw per day, respectively, on the basis of an increased incidence of hepatotoxicity in male and female mice and amyloidosis in female mice at 2000 mg/kg, equal to 274 and 392 mg/kg bw per day, respectively (MacKenzie, 1996c; Slone, 1997).

Groups of 50 male and 50 female Crl:CD-1®(ICR)BR mice were fed diets containing famoxadone (purity, 97.3%) at a concentration of 0, 2000, or 7000 mg/kg for approximately 18 months. These dietary concentrations provided doses equal to 0, 246 or 887 mg/kg bw per day for males, respectively, and 0, 348 or 1298 mg/kg bw per day for females, respectively. The study complied with EC Directive 87/302/EEC and was conducted according to the data requirements of the United States EPA pesticide assessment guidelines OPPTS 870-4200, OECD test guideline 451 and MAFF Japan Nohsan No. 4200. The objective of this study was to evaluate the tumourigenic potential of famoxadone in mice and not to establish an NOAEL in mice.

There were no treatment-related effects upon survival and the few clinical observations that were recorded were sometimes increased (incidence of pale mice and stained fur or skin), sometimes decreased (hair loss) in male and female mice at 7000mg/kg, and consequently were not considered to be related to treatment. Compound-related reductions in mean body weight, body-weight gain, and food use efficiency were observed in males at 7000mg/kg. Effects were primarily observed after the first three months of exposure. No adverse, treatment-related effects were observed on any of these parameters in males at 2000mg/kg and no effects on food consumption were observed in any group of males. There were no adverse, treatment-related effects in either mean body weight or body-weight gain in females of any group. Mean food use efficiency in females at 7000mg/kg, however, was below that for controls over the 18-month feeding period.

There were no compound-related effects on differential blood count observed in blood smears collected at 18 months from animals in the control group and in the group receiving the highest dose.

Ophthalmological examination was not performed, but microscopy of the eyes revealed no dose-related effects on the incidence of cataracts, these being of either mild or moderate severity in animals in the groups receiving famoxadone at 0, 2000 and 7000mg/kg, respectively: males, 9/50, 1/14 and 0/50; and females, 2/50, 0/15 and 0/50.

Treatment-related increases in liver weight (absolute and relative to body weight and brain weight) and hepatocellular hypertrophy were observed in almost all males and females at 2000 and 7000mg/kg, but these changes were considered to be non-adverse, physiologically adaptive responses to exposure to a xenobiotic. In males and females at 2000 or 7000mg/kg, there was also microscopic evidence of hepatotoxicity, including diffuse fatty change (females only: 4% and 16%, respectively, versus 0% in controls), increased lipofuscin pigment in Kupffer cells (males: 10% and 42%, respectively, versus 6% in controls; females: 22% and 26%, respectively, versus 8% in controls), individual hepatocellular necrosis; (males only: 4% and 14%, respectively, versus 0% in controls), erythrocytic inclusions in hepatocytes and increased mitotic figures (females only: 8% and 14%, respectively, versus 0% in controls).

A NOAEL was not identified in this study owing to effects on body weight, body-weight gain and food use efficiency in male mice and hepatotoxicity in mice of both sexes receiving famoxadone at a dietary concentration of 7000mg/kg (equal to 887 and 1298mg/kgbw per day, respectively, for males and females, respectively), the highest dose tested (MacKenzie, 2002).

The Meeting concluded that famoxadone is not carcinogenic in male or female mice.

Rats

Groups of 92 male and 92 female Crl:CD®BR rats were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 10, 40, 200 or 400mg/kg for approximately 23 months (males) or 24 months (females). These dietary concentrations provided doses equal to 0, 0.42, 1.62, 8.37 or 16.8mg/kgbw per day for males, respectively, and 0, 0.53, 2.15, 10.7 or 23.0mg/kgbw per day for females, respectively. The study complied with EC directive 87/302/EEC and was conducted according to the data requirements of the United States EPA pesticide assessment guidelines OPPTS 870-4200, OECD test guideline 451

and MAFF Japan Nohsan No. 4200. The objective of this study was to evaluate chronic toxicity and the tumourigenic potential of famoxadone in rats and to identify an NOAEL in rats. Ophthalmic observations were made on all rats before the start of the study and after approximately 12 and 22 months. Blood chemistry measurements and haematology were performed on 10 rats of each sex per group at approximately 3, 6, 12, 18 and 22 (males) or 23 (females) months. Studies of cell proliferation were made on livers of five rats of each sex per group at approximately 2 weeks and 12 months. β -Oxidation enzyme measurements were made on an additional five rats of each sex per group at these two time-points. Full pathological evaluation was carried out on 10 rats of each sex per group that were killed after approximately 12 months.

There were no treatment-related deaths and clinical signs were limited to an increase in the incidence of ruffled fur in males at 400 mg/kg. Biologically significant decreases in mean body weight and mean body-weight gain were observed in females at 400 mg/kg; final mean body weights were 82% of those of the control group, and body-weight gain over the 713 days of treatment was 75% of that of the control group. No body-weight effects were observed in the male rats.

There were indications of a mild, treatment-related induction of cataracts, although indirect ophthalmoscopy alone gave little indication of any problem. In males at 0, 10, 40, 200 and 400 mg/kg, respectively, the incidences of cataracts (Ca) and corneal opacities (Op) after 12 months were: 0/82, 0/79, 0/76, 0/76 and 1Ca + 1Op/78, while in males of the same groups after 23 months the incidences were: 1Op/24, 0/22, 1Ca + 1Op/31, 1Op/27 and 2Ca + 1Op/21. In females after 12 months the respective incidences were: 0/79, 1Op/82, 2Ca/77, 0/81, 1Ca/78, while after 24 months the incidences were: 1Ca/31, 0/29, 1Ca/23, 0/27, 2Ca/32. Microscopic examination of the eyes suggested more strongly that there might be an increase in cataracts. The incidences of diagnosed cataracts in males at 0, 10, 40, 200 and 400 mg/kg, respectively were: 3/62, 0/43, 1/34, 1/38 and 7/62. Of the seven microscopically diagnosed cataracts in the group receiving famoxadone at 400 mg/kg, three had been diagnosed ophthalmoscopically as such and one had been diagnosed as an opacity; the remaining three were not recognized *in vivo*. A subsequent re-evaluation of the eyes of the male rats concluded that the cataracts were not in fact cataracts but were lenticular degenerations (either unilateral or bilateral) and the total frequencies were 5/59, 4/56, 10/58, 4/60, 9/60 (Frame & Sykes, 1999).

No differences judged to be of any biological significance were observed in data on blood chemistry or urine analysis from any group. There were a few statistically significant changes in these parameters, but they were not toxicologically important, either because they were of small magnitude or because they failed to follow any monotonic response with increasing dose.

A mild regenerative, macrocytic, haemolytic anaemia was observed in both sexes at 400 mg/kg. In male rats of this group there were statistically significant reductions in erythrocyte counts, and increases in reticulocyte counts at 3, 6 and 12 months. At these same times, there were statistically significant increases in mean corpuscular volume and mean corpuscular haemoglobin. In female rats at 400 mg/kg, there were statistically significant reductions in erythrocyte counts, erythrocyte volume fraction and haemoglobin concentrations at 3, 6, 12 and 18 months, but increases in reticulocyte counts were observed only at 3 months. There was no evidence for a haemolytic effect in males at 18 or 22 months or in females at 23 months. No treatment-related effects on haematology were observed in rats

at ≤ 200 mg/kg. Morphological evidence of extravascular haemolysis and a regenerative response was observed microscopically at 400 mg/kg in both sexes at 1 year and included increases in splenic macrophage pigment (in males), hepatic Kupffer cell pigment (in females), splenic haematopoiesis (in males), and bone-marrow hyperplasia (in males). After 2 years, microscopic evidence of haemolysis was no longer evident; but a small increase in large spleens in males and females at 400 mg/kg was probably related to the haemolysis.

No significant effects on liver weight or weights of other organs were noted in this study. Treatment-related gross liver foci were observed in some males at 400 mg/kg. Histological evidence of hepatocellular toxicity was also found at this dose, in both male and female rats. At 1 year, there was an increase in focal cystic degeneration and focal hepatocellular degeneration in male rats at 400 mg/kg. After 2 years, there were increases in focal hepatocellular degeneration in both sexes at this dose. This degeneration, however, was less severe and the foci were of smaller size than was observed at the higher doses used in the 90-day study (800 and 1600 mg/kg), and consequently it was not associated with any increases in liver enzymes in the serum. A compound-related increase in the incidence, size, and severity of eosinophilic foci of hepatocellular alteration was also observed in male rats at 400 mg/kg at 2 years. These eosinophilic foci occasionally included or were associated with the aforementioned focal hepatocellular degeneration. The biological significance of eosinophilic foci is not clear, since it appears that many such foci do not progress to neoplasia and, indeed, they may regress if exposure to the inducing compound is stopped. A retrospective analysis of six 2-year studies of carcinogenicity, previously reported by the United States National Toxicology Program, concluded that the common, spontaneously occurring eosinophilic foci in liver appeared not to be generally useful as predictive indicators of liver carcinogenesis (Harada et al., 1989).

Non-adverse liver changes observed in males and females at 400 mg/kg included hepatocellular hypertrophy and apoptosis (in females only). The hypertrophy was often associated with increases in some cytoplasmic organelles. In this case, hepatic total cytochrome P450, which was measured at 2 weeks and 1 year, was only increased in females at 400 mg/kg at the 1-year time-point and is, therefore, indicative of proliferation of smooth endoplasmic reticulum. Liver cell proliferation was measured using the 5-bromo-2'-deoxyuridine labelling technique, which showed that, at 2 weeks in male rats at 400 mg/kg, labelling was increased to 9.6-fold the control value, but values for both males and females for this group were similar to those of controls at the 1-year evaluation. Hepatic β -oxidation activity, increases in which indicates peroxisomal proliferation, was elevated in males at 400 mg/kg only at the 1-year evaluation. After 2 years, hepatocellular apoptosis was increased slightly in female rats at 400 mg/kg. The increases in these liver effects at 400 mg/kg were all considered to be non-adverse effects related to the pharmacological response to a xenobiotic.

Neoplasms do not appear to have been induced by famoxadone. At 1 year, there was a statistically significant increase in adenomas of the pars distalis of the male pituitary at 400 mg/kg (0 mg/kg, 1/10; 400 mg/kg, 4/10). This result was not, however, substantiated by the data from the 2-year study in which the incidences for the same, very common tumour in this strain of rat were not increased by treatment (0 mg/kg, 49/62; 400 mg/kg, 41/62). Only for Leydig cell adenoma was a statistically significant increased incidence (according to a Cochran–Armitage test for trend) observed in the 2-year study. Incidences in the groups receiving famoxadone at 0, 10, 40, 200 and 400 mg/kg were: Leydig cell adenomas, 0/62, 0/62, 1/62, 1/61 and 3/62, respectively; and Leydig cell hyperplasia, 23/62, 21/62, 15/62,

20/62 and 19/62, respectively. A pair-wise comparison using the Fisher exact test (which is acceptable in this case, given the very similar patterns of mortality) of the groups receiving 0 and 400 mg/kg was not significant. Also, comparison by the Fisher exact test between the incidence for historical controls pooled from the six most appropriate studies (incidence, 9/351, range, 0–4.9%) and that for the group at 400 mg/kg was not significant ($p = 0.26$). Furthermore, there was no treatment-related increase in the incidence of hyperplasia of Leydig cells, from which adenomas are derived.

The NOAEL in male and female rats treated with diets containing famoxadone for 2 years was 200 mg/kg, equal to 8.4 and 10.7 mg/kg bw per day, respectively, on the basis of clinical signs (males), decreased body weights (females), mild hepatotoxicity, and mild regenerative haemolytic anaemia at 400 mg/kg, equal to 16.8 and 23.0 mg/kg bw per day, respectively, the highest dose tested (MacKenzie, 1996d; Sykes & Frame, 1997; Frame & Sykes, 1999).

The Meeting concluded that famoxadone is not carcinogenic in either rats or mice.

2.4 Genotoxicity

Famoxadone was tested for genotoxicity in a range of assays, both in vitro and in vivo (Table 5). There was no evidence of genotoxicity in vitro according to tests for gene mutation in bacteria (*S. typhimurium* and *E. coli*), gene mutation in Chinese hamster ovary cells and unscheduled DNA synthesis (two studies in primary cultures of rat hepatocytes). In a single study of chromosomal aberrations in cultures of human lymphocytes taken from two donors, significant increases in the proportion of abnormal cells were observed in two experiments conducted in the absence of an exogenous metabolic activation system, after incubation for 3 h. Significant responses were observed at a concentration of 20 and 25 µg/ml

Table 5. Results of studies of genotoxicity with famoxadone

End-point	Test object	Dose (LED/HID)	Purity (%)	Result	Reference
<i>In vitro</i>					
Gene mutation	<i>S. typhimurium</i> strains TA100, TA1535, TA1537, TA1538, TA98; <i>E. coli</i> WP2 <i>uvrA</i>	5000 µg/plate	97.4	Negative ^b	Bentley (1995)
Unscheduled DNA synthesis	Male Crl:CD®BR rat primary hepatocytes	7.5 µg/ml	97.4	Negative ^b	Bentley (1994)
Unscheduled DNA synthesis	Male Crl:CD®BR rat primary hepatocytes	5.0 µg/ml	97.4	Negative ^b	Cifone (1999b)
Gene mutation	Chinese hamster ovary cells, <i>Hprt</i> locus	600 µg/ml	97.3	Negative ^b	Cifone (1999a)
Chromosomal aberration	Human lymphocytes	15 µg/ml ± S9 30 µg/ml + S9	97.4	Positive Negative ^b	Gerber (1995)
<i>In vivo</i>					
Unscheduled DNA synthesis in vivo/in vitro	Male Crl:CD®BR rat liver cells	2000 mg/kg bw per os × 1	97.4	Negative	Fellows (1998)
Micronucleus formation	Male Crl:CD®BR rat, bone-marrow cells	20 000 mg/kg in diet, 14 days	>98	Negative	MacKenzie (1992)
Micronucleus formation	Male Crl:CD-1®(ICR)BR mouse, bone-marrow cells	5000 mg/kg bw, per os × 1	97.4	Negative ^b	Kuykendall (1994)

LED, lowest effective dose; HID, highest ineffective dose
S9, 9000 × g supernatant fraction of rat liver

in the first experiment and at 15, 20 and 25 µg/ml in the second experiment. Most of the damage consisted of chromatid gaps, but chromatid breaks were also frequent. The frequency of chromosomal damage was not significantly elevated, but occasional chromatid triradials and quadriradials (i.e. complex exchanges) were observed in treated cells, whereas they did not occur in untreated cells of either experiment (although these rare exchange figures were also found in the vehicle control culture from one donor in the presence of a metabolic activation (S9, 9000 × g supernatant fraction of rat liver). In the presence of a metabolic activation system, again with an incubation of 3 h, there were no indications of clastogenic activity with famoxadone at concentrations of up to 30 µg/ml. This loss of activity could be caused either by metabolism to an inactive product or by competitive binding to the added proteins in the incubation mixture, thereby reducing the available concentration of famoxadone available for reaction with the target cells. No genotoxic activity was observed in three experiments conducted in vivo. One of these was a study of unscheduled DNA synthesis in vivo/in vitro in the liver of male Crl:CD®BR rats either 2–4 or 14–16 h after administration of famoxadone as a single oral dose at 0, 800 or 2000 mg/kgbw by gavage. The other two studies were tests for micronucleus formation in bone-marrow cells of mice. In the first of these, groups of Crl:CD-1®(ICR)BR mice were given famoxadone as a single oral dose at 0, 1250, 2500 or 5000 mg/kgbw by gavage. Mice receiving the highest dose (six of each sex per time-point) and the vehicle control (five of each sex per time-point) were killed at approximately 24, 48 and 72 h after treatment. Mice in the remaining two groups (five of each sex) were killed 24 h after dosing. No increases in the frequency of micronucleated polychromatic erythrocytes were observed in either sex at any dose. The second test for micronucleus formation was an addendum to a 14-day study of toxicity described above. Groups of Crl:CD®BR rats (five of each sex per group) were fed diets containing famoxadone (purity, >98%) at a concentration of 0, 6000, or 20 000 mg/kg, for 14 days. Bone-marrow smears were prepared from four rats of each sex per group and examined for micronuclei. No statistically significant increases in micronucleated polychromatic erythrocytes were observed and there was no change in the ratio of polychromatic erythrocytes to normochromatic erythrocytes.

Thus, the single indication of clastogenic activity observed in vitro was not confirmed in vivo. The Meeting concluded that famoxadone is unlikely to be genotoxic.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of 30 male and 30 female Crl:CD®BR rats were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 20, 200 or 800 mg/kg, equal to 0, 1.14, 11.3 and 44.7 mg/kgbw per day in males and 0, 1.45, 14.2 and 53.3 mg/kgbw per day in females in a two-generation study of reproduction, which was performed according to EC directive 87/302/EEC and the data requirements of the United States EPA pesticide assessment guidelines subdivision F 83-4, OECD guidelines for testing of chemicals section 4, No. 416 and MAFF testing guidelines for toxicology testing NohSan 59 No. 4200. After receiving the test diets for at least 70 days, the animals were mated. Thirty offspring (F₁) of each sex per group were fed diets containing the same concentrations of famoxadone for at least 105 days after weaning, before mating to produce a second generation (F₂). Twenty F₁ and F₂ weanlings of each sex per group that had not been selected to produce the next generation were selected for gross examination post mortem. The remaining weanlings were killed

without examination. Before mating, measurements of blood chemistry were made on selected adult rats (10 of each sex per group) in both generations. After the litters were weaned, all parental rats were killed and gross examinations were conducted post mortem. In particular, livers of all adults were weighed and peroxisomal β -oxidation activities measured in livers from five rats of each sex per group. Some liver samples were lost, so these numbers were reduced to four per group in P_0 males and F_1 males and females at 200 mg/kg. Tissues from rats of both generations receiving famoxadone at 0 and 800 mg/kg were examined microscopically, as were any gross lesions and target organs from adult rats in all groups.

No adverse treatment related effects were observed among rats at ≤ 200 mg/kg.

At 800 mg/kg, a number of treatment-related and statistically significant effects were recorded. There were, however, no treatment-related effects on survival. There were increased incidences of diarrhoea in P_1 males and of alopecia in F_1 females during the period before mating, and in P_1 and F_1 females during gestation. Mean body weights, overall mean body-weight gains and overall mean food consumption was reduced in P_1 and F_1 males and females. Overall mean food use efficiency was also reduced for P_1 males and females during the period before mating. During gestation, mean body weight and overall mean food consumption was reduced for P_1 and F_1 females. Mean body weight was also reduced on days 0, 7 and 14 of lactation for P_1 and F_1 females.

There were no compound-related gross lesions, and the histology of the reproductive organs was unremarkable. There were, however, increases in the activities of hepatic enzymes measured in serum, as well as increases in serum bilirubin concentrations, which was indicative of hepatocellular injury and cholestasis in P_1 and F_1 males. Significant reductions in mean triglyceride concentrations in serum of P_1 and F_1 males and females and significant increases in mean cholesterol concentrations in serum of P_1 males and F_1 males and females suggested altered lipid metabolism. Within samples of liver itself, the hepatic β -oxidation rate was increased for P_1 and F_1 males and females. It is noted that these liver effects were similar to those observed in females at 800 mg/kg in the 90-day feeding study in rats, described above. Also, as in the 90-day study, the increase in serum liver enzymes was much greater in male than in female rats, correlating with a decrease in mean liver weights in male rats, rather than an increase as seen in female rats. Histological examination did not indicate hepatotoxicity.

Reproductive indices were unaffected by treatment at any dose. These indices included mating, fertility and gestation indices as well as the indices that describe pup viability at parturition, and on postnatal days 4 and 21. The only recorded effect of treatment on the pups was a reduction in mean pup weight throughout the 21-day period of lactation for F_1 litters (average reductions, 7–9%) and from postnatal day 4 until the end of lactation for F_2 litters (average reductions, 6–8%), these effects occurring at 800 mg/kg.

In the multigeneration study of reproduction in rats treated with famoxadone, the NOAEL for adult rats and their offspring was 200 mg/kg, equal to 11.3–14.8 and 14.2–17.5 mg/kgbw per day in adult males and females, respectively, on the basis of maternal and paternal systemic toxicity (decreased body-weight gains and food consumption, and hepatotoxicity) at 800 mg/kg, equal to 44.7–62.1 mg/kgbw per day. The NOAEL for offspring toxicity was 200 mg/kg, on the basis of reduced pup weight gain at 800 mg/kg. There were no effects on reproductive indices at concentrations up to and including 800 mg/kg,

the highest dose tested (Kreckmann, 1995). The Meeting concluded that famoxadone is not a reproductive toxicant in rats.

(b) Developmental toxicity

Rat

In a study of developmental toxicity, groups of 25 time-mated, female Crl:CD®BR rats were given famoxadone (purity, 97.4%) in corn oil by gavage at a dose of 0, 125, 250, 500, and 1000 mg/kg bw per day on days 7–16 of gestation. All rats survived to the end of the study and no internal lesions were found at autopsy. Dams were killed on day 22 of gestation and subjected to gross examination. Fetuses were removed, weighed, sexed, and subjected to external, visceral, and skeletal examination.

Maternal effects were limited to a decrease in mean food consumption and body-weight gains at 500 and 1000 mg/kg bw per day during days 7–9 of gestation. At 1000 mg/kg bw per day, there was a recovery in food consumption on days 17–22 of gestation, when it was significantly increased. No compound-related effects were observed in any reproductive or developmental end-points at any dose.

The NOAELs in the study of developmental toxicity in rats were 250 mg/kg bw per day for the dams, on the basis of decreases in food consumption and body-weight gains at 500 mg/kg bw per day, and 1000 mg/kg bw per day, the highest dose tested, for the fetuses (Murray, 1994; Munley, 1999a). The Meeting concluded that famoxadone is neither a teratogen nor a developmental toxicant in rats.

Rabbits

In a study of developmental toxicity, groups of 20 time-mated, female Hra: (NZW)SPF rabbits were given famoxadone (purity, 97.4%) suspended in 0.5% Tween 80 at an oral dose of 0, 100, 350, or 1000 mg/kg bw per day by gavage on days 7–19 of gestation. Dams were killed on day 29 of gestation, or after aborting, and subjected to gross examination. Fetuses were removed, weighed, sexed, and subjected to external, visceral, and skeletal examinations.

Four out of the 17 pregnant rabbits receiving a dose of 1000 mg/kg bw per day suffered severe weight loss, decreased food consumption, faecal impaction, and subsequent abortions that occurred during days 19–23 of gestation. All these effects were considered to be secondary to gastrointestinal impaction with the dosing suspension, due to its high viscosity. In one of these four rabbits receiving the highest dose, trichobezoar (ball of hair in the stomach or intestines) was indicated by examination post mortem and may have exacerbated the impaction. Rabbits without signs of impaction had neither signs of maternal toxicity nor abortions; consequently, famoxadone was considered not to have produced systemic toxicity at up to and including a dose of 1000 mg/kg bw per day. One rabbit receiving a dose of 100 mg/kg bw per day aborted without showing any of the signs seen at a dose of 1000 mg/kg bw per day. This abortion was considered to be a chance occurrence; this conclusion was supported by the absence of abortions at 350 mg/kg bw per day. In a second case of abortion at 100 mg/kg bw per day, the animal experienced similar reductions in body-weight gain and food consumption as recorded for those cases in animals receiving a dose of 1000 mg/kg bw per day. There appears to be some doubt regarding the cause of this abortion, but examination post mortem did reveal white fibrous material (probably hair) in the

stomach contents, which perhaps suggests that trichobezoar was also a preliminary cause of this abortion.

No compound-related effects were observed in any reproductive or developmental end-point at any dose. The maternal and fetal NOAEL in this study of developmental toxicity in rabbits was 1000 mg/kg bw per day, the highest dose tested (Munley, 1999b; Munley, 1999c).

The Meeting concluded that famoxadone is not teratogenic and does not present a developmental hazard in rabbits.

2.6 *Special studies*

(a) *Neurotoxicity*

In a study of neurotoxic potential, groups of 12 male and 12 female CrI:CD®BR rats were given famoxadone (purity, 97.4%) as a single oral dose at 0, 500, 1000 or 2000 mg/kg bw, and were observed for 15 days. Neurobehavioural testing, consisting of FOB and motor activity monitoring, was conducted on all rats before treatment (baseline), on day 1 approximately 1–3 h after treatment, and on days 8 and 15. At the end of this period, six rats of each sex per group were perfused with fixative, and samples were taken of nervous and muscle tissue (brain, spinal cord, sciatic/tibial nerves, ganglia, cervical and lumbar dorsal and ventral root fibres and ganglia and gastrocnemius muscle). Only the tissues from the controls and rats in the group receiving the highest dose were examined histologically.

No treatment-related general toxicological effects were observed in the groups given famoxadone at a dose of 500 or 1000 mg/kg bw. Male rats (but not females) at 2000 mg/kg bw group had significantly lower body-weight gain and food consumption over days 1–2. During the remainder of the test period, there were no treatment-related effects on body-weight gain or food consumption values in any group. There were no adverse effects on survival, clinical signs or indicators of neurotoxicity at any dose. An increase in the incidence of palpebral (eyelid) closure during day 1 of FOB monitoring, together with decreased body-weight gain and food consumption in males at 2000 mg/kg bw was interpreted as general malaise. Also, a significant reduction in the number of movements in the motor activity assessment on day 15 in males at 1000 mg/kg bw was not considered to be treatment-related, in view of the absence of any dose–response relationship. No treatment-related pathological effects were observed in this study.

The NOAELs in this assessment of acute neurotoxicity were 1000 mg/kg bw in males and 2000 mg/kg bw in females, on the basis of lower body-weight gain and food consumption and higher incidence of palpebral closure in males at the limit dose of 2000 mg/kg bw and the absence of any effects in females (Malley, 1995a).

In a study of neurotoxic potential, groups of 12 male and 12 female CrI:CD®BR rats were given diets containing famoxadone (purity, 97.4%) at a concentration of 0, 50, 200 or 800 mg/kg for 90 days. These dietary concentrations provided doses equal to 0, 2.9, 11.7 and 46.9 mg/kg bw per day in male rats and 0, 3.7, 14.4 and 59.3 mg/kg bw per day in female rats. Clinical signs, body weights, and food consumption were recorded during the treatment period. Neurobehavioural testing, consisting of FOB and motor activity monitoring, was conducted on all rats before treatment (baseline), on day 1 approximately 1–3 h after compound administration, and in weeks 4, 8 and 13. At the end of this period, six rats of each sex per

group were perfused with fixative and samples were taken of nervous and muscle tissue (brain, spinal cord, sciatic/tibial nerves, gasserian ganglia, cervical and lumbar dorsal and ventral root fibres and ganglia and gastrocnemius muscle). Only the tissues from rats in the control group and the group receiving the highest dose were examined histologically.

Both male and female rats at 800mg/kg had decreased body-weight gains, food consumption, and food use efficiency. There were no compound-related effects detected in clinical observations, neurobehavioural evaluations, motor activity, or nervous system morphology. Therefore, evidence of neurotoxicity was not observed at any dose. The NOAEL for male and female rats in this 90-day study of neurotoxicity was 200mg/kg, equal to 11.7 and 14.4mg/kgbw per day, respectively, on the basis of general toxicity observed at 800mg/kg, equal to 46.9 and 59.3mg/kgbw per day, respectively (Malley, 1995b).

(b) *Immunotoxicity*

Studies were conducted in mice and rats to evaluate the potential of famoxadone to suppress the primary humoral immune response to sheep erythrocytes. Groups of 10 male and 10 female Crl:CD-1®(ICR)BR mice were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 50, 350, 2000, or 7000mg/kg for 28 days. These dietary concentrations provided doses equal to 0, 8, 55, 327 or 1186mg/kgbw per day in male mice and 0, 11, 72, 417 or 1664mg/kgbw per day in female mice. Groups of 10 male and 10 female Crl:CD®(SD)IGS BR rats were fed diets containing famoxadone (purity, 97.4%) at a concentration of 0, 50, 100, 200, or 800mg/kg. These dietary concentrations provided doses equal to 0, 4, 7, 14 or 55mg/kgbw per day in male rats and 0, 4, 8, 16 or 57mg/kgbw per day in female rats. Body weights, food consumption, and clinical observations were recorded during the test period. To evaluate the primary humoral immune response, all animals were injected intravenously on day 23 (mice) or day 22 (rats) with sheep erythrocytes and killed on test day 28. The spleen and thymus were weighed, and serum was analysed for sheep erythrocyte-specific immunoglobulin M (IgM) antibody.

In mice, there were no effects on body weight, body-weight gain, food consumption, food use efficiency, clinical signs of toxicity, or mortality at any dietary concentration. There were also no treatment-related effects on spleen weights of male mice or thymus weights of male and female mice. Increased spleen weights (29%) were observed in females at 7000mg/kg, but these were considered secondary to the haematological effects, which although not a subject of this study, had been observed in previous studies. A small but statistically significant decrease in the primary humoral immune response to sheep erythrocytes occurred in male mice at 7000mg/kg. Although the biological significance of the lowered humoral response was equivocal, the effect was considered to be indicative of minimal immunotoxicity. Famoxadone did not affect the primary humoral response to sheep erythrocytes in female mice at any dietary concentration tested. The NOAEL for immunotoxicity in mice was 2000mg/kg for males, equal to 327mg/kgbw per day, and 7000mg/kg for females, equal to 1664mg/kgbw per day. The NOAEL for systemic toxicity, other than immunotoxicity, was 7000mg/kg for males, equal to 1186mg/kgbw per day, the highest dose tested, and 2000mg/kg for females, equal to 417mg/kgbw per day, on the basis of increased spleen weight at 7000mg/kg, equal to 1664mg/kgbw per day.

In rats, there was no evidence of immunotoxicity observed in either males or females at any dietary concentration. Decreased body-weight gain (males, 24%; females, 49%), food consumption (males, 13%; females, 18%) and food use efficiency and increased spleen

weights (males, 17%; females, 8%) were observed in rats at 800 mg/kg. The increased spleen weights were considered to be secondary to the haematological effects, which, although not a subject of this study, had been observed in previous studies. The NOAEL for immunotoxicity in rats was 800 mg/kg in males and females, equal to 55 and 57 mg/kg bw per day, respectively, the highest dose tested. The NOAEL for systemic toxicity, other than immunotoxicity, was 200 mg/kg in males and females, equal to 14 and 16 mg/kg bw per day, respectively, on the basis of decreases in body-weight gain, food consumption and food use efficiency and increased spleen weights in rats fed famoxadone at a dietary concentration of 800 mg/kg, equal to 55 and 57 mg/kg bw per day (Ladics, 1999a, 1999b).

(c) *Pharmacological study in mice and rats*

A study was conducted to evaluate the potential pharmacological activity of famoxadone. Groups of 3–10 male Crj:CD-1(ICR) mice or male Crj:Wistar rats were given a single oral dose of famoxadone (purity, 97.0%) at 0, 500, 1500 or 5000 mg/kg bw in corn oil and acetone (85:15). The study was conducted according to Japanese guidelines on agricultural chemicals (1985) and, although the study was not conducted to any particular GLP guidelines, the standard operating procedures of the performing laboratory (Mitsubishi Chemical Safety Institute) were followed.

The animals were observed for effects on the central nervous system (general behaviour assessment), hexobarbital sleeping time, synergistic effect on convulsions following electrical shock, body temperature, cardiovascular system (heart rate, blood pressure), autonomic nervous system (pupil size), gastrointestinal tract (intestinal propulsion), skeletal muscle (traction test), and blood coagulation (prothrombin and activated partial thromboplastin times).

A decrease in locomotor activity during the general behavioural observation period was noted. No significant effects were observed on hexobarbital sleeping time, subthreshold electrical shock, or body temperature. Soft faeces were noted at 500 and 1500 mg/kg bw. As the main component of the vehicle was corn oil, which often induces soft faeces, this effect was attributed to the solvent; however, animals in the control group received the same vehicle. Blood pressure and heart rate, pupil size, intestinal propulsion, traction, and blood coagulation times were not affected by treatment with famoxadone. The conclusion of the study was that there was no clear pharmacological effect produced by famoxadone at up to and including the highest dose tested, 5000 mg/kg bw (Horii, 1997).

(d) *Reversibility of effects on erythrocyte mass parameters in rats*

In studies in rats receiving repeated doses, described above, exposure to famoxadone at dietary concentrations of ≥ 400 mg/kg produced a mild haemolytic anaemia, characterized by reductions in erythrocyte count, haemoglobin concentration, and erythrocyte volume fraction. On the basis of the reticulocytosis and bone-marrow hyperplasia observed in these studies, the anaemia is considered regenerative. In an attempt to confirm the regenerative nature of the observed anaemia, a study was conducted to evaluate the potential reversal of the effects when exposure to famoxadone ceases.

Groups of 10 female Crl:CD®(SD)IGS BR rats were given diets containing famoxadone (purity, 97.3%) at a concentration of 0 or 800 mg/kg for 35 days, and then basal diet only until day 58, when the study was terminated. These dietary concentrations provided doses equal to 0 or 61.6 mg/kg bw per day. Body weight, food consumption, and clinical

signs were evaluated weekly. Erythrocyte mass parameters (erythrocyte counts, erythrocyte volume fraction and haemoglobin concentrations) were evaluated at 16 days after first exposure to famoxadone, and every 2 weeks thereafter. Once toxicologically significant reductions were observed, the diet of the rats receiving famoxadone was replaced with control diet. Evaluation of erythrocyte counts, erythrocyte volume fraction and haemoglobin concentrations continued until all values returned to control levels. This study complied with United States EPA FIFRA (40 CFR, part 160) and/or EPA TSCA (40 CFR, part 792) standards for GLP, which are consistent with the OECD principles of GLP (as revised in 1997) published in ENV/MC/CHEM(98)17, and MAFF Japan good laboratory practice standards (59 NohSan No.3850).

After 30 days of exposure to famoxadone at 800 mg/kg, a mild anaemia was produced. The three erythrocyte-mass parameters were reduced by 11–16% below control values. The diet of the rats receiving famoxadone was replaced with the control diet on day 35. Nine days later, the mean erythrocyte volume fractions and haemoglobin concentrations in this group were slightly higher than those for the control rats. By the next time-point at which evaluation of blood parameters was made, after 23 days of exposure to control diet, full recovery from the anaemia had occurred. Erythrocyte counts, erythrocyte volume fractions and haemoglobin concentrations were similar to or greater than the values for the control group at this time.

No compound-related effects on mortality or clinical signs were produced. Lower body weight, body-weight gain, food consumption, and food use efficiency were observed by day 7 in female rats at 800 mg/kg. Once exposure to famoxadone was stopped, no further reductions in these parameters occurred and, during this recovery phase, body-weight gain and food use efficiency values rose above those of the controls (MacKenzie, 2000).

(e) *Test for cytotoxicity in lens epithelial cells in vitro*

A test for cytotoxicity in vitro with famoxadone (purity, 97.4%) was conducted using primary cultures of canine and primate (mostly rhesus monkey, but not always identified) lenticular epithelial cells, an immortalized mouse lens epithelial cell line (NK-35), and an immortalized human corneal epithelial cell line (SV-40). Cultures were exposed to famoxadone at eight concentrations, ranging from 100 pg/ml to 1 mg/ml, for 3, 24 or 48 h. Cytotoxicity was measured using a dual colour viability assay, which simultaneously assessed intracellular esterase activity and plasma membrane integrity.

Cytotoxicity was demonstrated in all cell cultures, irrespective of the species from which they were derived, when they were exposed to famoxadone at 1 mg/ml for 3, 24, or 48 h. No biologically significant effects were noted below this dose. Overall there were no clear differences observed in the susceptibility of the four cell cultures to cytotoxicity induced by famoxadone (Murphy, 1997).

(f) *Other toxicological information*

(i) *Significance of haematological effects*

Multiple exposures to famoxadone produced mild haematological changes, consistent with haemolysis, that were observed in rats, dogs and cynomolgus monkeys, but were less obvious in mice, particularly males. Haematological changes were frequently the basis for the NOAELs.

Functionally, anaemia is defined as a decrease in erythrocyte mass parameters (erythrocyte count, haemoglobin concentration, and erythrocyte volume fraction) to below normal levels (Farver, 1989; Erslev, 1990; Duncan et al., 1994; Sodikoff, 1995; Sasse, 1996). Typically, anaemia is classified within two broad categories—regenerative or non-regenerative—on the basis of the bone-marrow response. In regenerative anaemia, the bone marrow is actively responding by increasing the production of erythrocytes. Evidence of a bone-marrow response is seen as an increase in circulating young erythrocytes (reticulocytes) and an increase in erythropoiesis in bone marrow. In a non-regenerative (aplastic) anaemia, on the other hand, the bone marrow is unable to respond to the anaemic state, owing to reduced or defective erythropoiesis.

The consequence of anaemia is reduced delivery of oxygen to the tissues, an indicator of which is a change in the oxygen–haemoglobin dissociation curve. In humans, this change occurs when the concentration of haemoglobin decreases by about 30–40% (Stehling & Simon, 1994). Similarly, in dogs with experimentally-induced anaemia, transport of oxygen to tissues is not significantly altered until the erythrocyte volume fraction is decreased by about 30% (Fan et al., 1980). Therefore, adverse effects on the organism do not occur until erythrocyte parameters are depressed to a significant degree. Critical decreases in the concentration of haemoglobin and/or erythrocyte volume fraction were not reached in the studies reported here. It could be suggested, therefore, that the NOAELs proposed are conservative. However, it is well known that there are species differences in the sensitivity of erythrocytes to potentially haemolytic agents. While comparisons of the effects in vitro of a chemical (and its identified mammalian or plant metabolites) on erythrocytes from different species, including humans, are relatively easy to perform, such comparisons have not been performed with famoxadone. Consequently, at this stage, it is not possible to conclude with any confidence that the NOAELs identified on the basis of anaemia are indeed conservative when making the interspecies extrapolation to humans.

(ii) *Significance of lens effects in studies in dogs treated with repeated doses*

The lens does not contain pigment or blood vessels, which would decrease its transparency. Consequently, the lens derives almost all its metabolic requirements from the aqueous humour, which is also the medium through which systemically available xenobiotics are delivered to the lens. Any opacity of the lens and its capsule is termed a cataract. Lenticular opacities may result from numerous different mechanisms (Brown & Bron, 1996), but for the vast majority of chemicals that induce cataracts these are unknown. Chemicals with widely differing structures and pharmacological activities have been reported in the literature to cause cataract in laboratory animals. In a survey of ocular toxicological profiles, the correlation between toxicity in rodent and in non-rodents was not established; few compounds are known to cause cataracts in both rats and non-rodents (Heywood, 1981, 1983). Since the toxic effects of various substances on the lens are quantitatively very different in different species, extrapolation of experimental animal data to humans must be done with caution.

Mechanisms leading to lens opacity include: changes in osmotic pressure leading to an increase in water content, denaturation of lens proteins such as crystallins (which are critical for lens clarity), and generation of reactive oxygen radicals resulting in oxidative damage (Basher & Roberts, 1995; Clang & Aleo, 1997). The normal lens is in a “dehydrated” state. This state strongly depends on the proper functioning of the ion and water

pumps that derive their energy from ATP delivered by lens epithelium and superficial lens fibres. The lens osmolarity is maintained by cations (sodium, Na^+ and potassium, K^+) and anions (chloride, bicarbonate, sulfate, ascorbate, glutathione, acidic groups of lens proteins and glycoproteins). The observation that the study in dogs fed with high dietary concentrations of famoxadone (1000 reduced to 600 mg/kg) resulted in an increase in serum K^+ , indicating an effect on ion homeostasis at toxic doses, could therefore have a bearing on cataract formation. However, the increase in K^+ was significant only at this high dose, while lenticular damage was also found in dogs given diets containing famoxadone at 300 mg/kg. Another general mechanism implicated in cataract formation is activation of calcium-dependent proteases (calpains) by various agents (Clang & Aleo, 1997). Alterations in calcium homeostasis by a variety of mechanisms could, therefore, produce cataracts. There are no indications, however, that this happens in dogs treated with famoxadone.

The known biochemical mechanism of action of famoxadone is inhibition of the mitochondrial respiratory chain at complex III (ubiquinol: cytochrome c oxidoreductase), resulting in decreased production of ATP by the cell. ATP is essential for maintenance of cellular metabolism and the function of ATP-dependent cellular enzymes, in particular the Na^+ - K^+ -ATPase that is critical for maintenance of normal cellular hydration. Therefore, reduction in cellular ATP could result in an increase in lens hydration and subsequent cataract formation. It is likely that this mechanism would require famoxadone to be present in the eye at above a certain threshold concentration. However, studies of distribution indicate that the concentrations likely to be found in the eye of a dog are low (Thalacker, 1996), while comparative data from other species are not available. Whether the concentrations would be sufficient to uncouple oxidative phosphorylation in the dog eye seems unlikely, since there is no evidence of mitochondrial toxicity *in vivo* in other organs in which the concentration of famoxadone may be 30–40-fold higher. However, metabolic studies conducted with famoxadone in rats and dogs demonstrated a longer half-life for radioactivity (parent and/or metabolite) in the dog, but no major qualitative differences in metabolic profile were found and the quantitative differences were small.

In addition, ATP is required to maintain the reducing power of the cell, thus a decrease in ATP (unlikely as this may be) would increase susceptibility to oxidative stress, which is also implicated in cataract formation. Dogs have been demonstrated to be relatively deficient (compared with primates) in numerous mechanisms that protect against oxidative stress. Thus, in an interspecies comparison of glutathione (GSH) reductase activity in the lens, carnivores were found to have the lowest GSH reductase activities, while non-human primates (including cynomolgus monkeys) and humans had the highest activities (Rathbun et al., 1986). GSH reductase is the rate-limiting enzyme in the GSH redox cycle, which can protect cells against oxidative damage, particularly from hydroperoxides. Hence cells with lower activities of this enzyme will be in a more vulnerable position, should such substances be produced. These lower activities in dogs may explain the observed increased sensitivity of this species to lens toxicity after exposure to famoxadone, but they do not render the dog unique.

Ascorbic acid can also act as a reducing agent, thereby protecting the cell against oxidative reactions initiated by oxygen and free radicals. It is present at unusually high concentrations in primate, including human, eyes and can be found in the cornea, aqueous humour, lens, vitreous humour and retina. Humans and monkeys contain identical concentrations of ascorbate in their aqueous humour and lens, (1.0 and 1.25 mmol/l, respectively). In comparison, the concentrations of ascorbate in the aqueous humour and lens of dogs are

0.31 and 0.2 mmol/l, respectively. In one study, the concentration of ascorbate in the aqueous humour of human patients with cortical cataracts was significantly lower than in that of unaffected patients, suggesting that low ascorbate might predispose to cataract formation by reducing the antioxidant function in the lens (Varma & Richards, 1988).

Exposure to famoxadone resulted in lens opacities and cataracts in outbred beagle dogs. These lesions (generally bilateral in occurrence) were observed after exposure to low doses of famoxadone in 90-day and 1-year studies. Exposures of similar and longer durations did not produce cataracts in mice or cynomolgus monkeys, despite exposure at much higher doses. Microscopically, however, cataracts were identified in the 18-month study in mice treated orally and in the 24-month study in rats treated orally. In mice there was clearly no dose-response relationship and in rats the excess incidence among males occurred at 40 and 400 mg/kg, but not at 200 mg/kg.

An additional review of the animal data and microscopic examination of eye sections from the studies in dogs treated with famoxadone was undertaken (Heywood, 1999); this review reached the same conclusions as those made by the original investigators, namely, that cataracts were induced by famoxadone in beagle dogs, but not in mice, rats or cynomolgus monkeys.

There are no qualitative differences between mammalian species and strains with respect to the differentiation process of lens fibres. Thus, there is not sufficient support at the moment to be able to conclude that the observed posterior subcapsular cataracts, most of which developed after 2–3 months treatment, and equatorial lens opacities that developed after 6–12 months treatment, are species-specific and restricted to beagle dogs. Such a conclusion would require a full understanding of the mechanism of action of famoxadone on the eyes in the various species and knowledge of the concentration of famoxadone in the aqueous humour and the lens.

The absence of effects in monkeys may be due in part to the relatively short period of exposure relative to the lifespan of monkeys, or to use of non-toxic doses of the test substance. The period of exposure might have been too short to lead to manifest equatorial and posterior subcapsular changes that were detectable using routine clinical and histopathological methods. It is noted that cynomolgus monkeys seem generally to be less sensitive to the toxic effects of famoxadone; the only observed effect was a mild anaemia at 1000 mg/kgbw per day. With regard to the presence or absence of treatment-related increases in cataracts in rats, it is noted that there is evidence that albino and pigmented rats have different sensitivities to cataractogenic factors, albino rats being less sensitive (Wegener & Eiben, 1992; Eiben & Wegener, 1995).

3. Observations in humans

No information was available.

Comments

Studies in rats show that about 40% of the administered dose of radiolabelled famoxadone is absorbed and rapidly eliminated from the body in the faeces (>75% in 24 h) and urine (about 10% in 24 h). Most of the administered dose found in the faeces is unmetabolized famoxadone. In rats, absorption from the gastrointestinal tract becomes the limiting

factor for internal exposure at doses greater than about 800 mg/kg bw. It appears that there are no important differences in the metabolism of famoxadone between dogs and rats, within the limits imposed by the different doses used, and that there were no significant differences between male and female rats (only males having been used in the experiments with dogs). The primary metabolic pathway involved the hydroxylation of the parent molecule to the corresponding mono- and dihydroxylated derivatives, which were only recovered from the faeces. Metabolites resulting from the cleavage of the oxazolidinedione ring moiety were recovered from the urine. A sulfate was the major urinary metabolite containing the phenoxyphenyl moiety, whereas 4-acetoxylaniline was the major urinary metabolite containing the phenylamino moiety. No parent famoxadone was detected in the urine.

Famoxadone has low acute toxicity when administered by oral, dermal, and inhalation routes. The acute LD₅₀ after oral administration is >5000 mg/kg bw in rats and the LD₅₀ after dermal administration is >2000 mg/kg bw in rabbits. The LC₅₀ in rats after 4 h is >5300 mg/m³, the only concentration tested. Famoxadone produces transient mild dermal irritation and transient mild ocular irritation, but does not cause skin sensitization.

In short-term studies of oral administration in rodents, dogs and non-human primates, and in long-term studies of oral administration in rodents, NOAELs for famoxadone were identified on the basis of effects on body weight and nutrition, mild haemolytic anaemia, and/or mild to moderate liver toxicity. Mild regenerative haemolytic anaemia was found in rats, mice, dogs and monkeys, as indicated by decreased erythrocyte counts, haemoglobin and/or erythrocyte volume fraction, increased reticulocyte counts, or other related changes in haematological parameters. Methaemoglobin formation was not measured. Secondary effects of anaemia were also found in the spleen (e.g. increased spleen weight, deposition of haemosiderin pigment, extra-medullary haematopoiesis), in the bone marrow (compensatory erythropoiesis), and in the liver (increased Kupffer cell pigment, increased bile pigment). In studies involving repeated dosing, anaemia was found to occur early in the study and often appeared to be compensated for later. In an experiment in which rats were fed famoxadone as a single dose at 800 mg/kg, equal to 61.6 mg/kg bw per day, blood samples were taken at multiple time-points. Mild anaemia was observed after 30 days, but not after 16 days. Famoxadone also induced hepatocellular responses that are normally considered to be adaptive (e.g. enlarged livers, increased liver weights and liver:body-weight ratios, hepatocellular hypertrophy). These adaptive responses were characterized by increased quantities of cytochrome P450 and/or increased rates of peroxisomal β -oxidation. Hepatotoxicity, which was mild, was observed only at higher doses and was characterized by mild histopathological lesions (e.g. single cell or focal necrosis, hepatocellular degeneration, diffuse fatty change, eosinophilic foci) and marginally elevated concentrations of blood enzymes suggestive of liver damage. The NOAELs after short-term oral administration were 62.4 mg/kg bw per day in mice treated for 3 months, 13 mg/kg bw per day in rats treated for 3 months, 1.2 mg/kg bw per day in dogs treated for 1 year and 100 mg/kg bw per day in cynomolgus monkeys treated for 1 year.

Long-term studies in rats (2 years) and mice (18 months) show little evidence of irreversible organ toxicity, although there was an increased incidence of generalized amyloidosis among female mice receiving famoxadone in the diet. Other effects that were observed, some of which formed the basis for the NOAELs, were reductions in body-weight gain, hepatotoxicity and mild regenerative anaemia. The NOAELs for long-term toxicity were 700 mg/kg, equal to 96 mg/kg bw per day, in mice, and 200 mg/kg, equal to 8.4 mg/kg bw per day, in rats. There was no evidence of carcinogenic potential with famoxadone at

doses up to the highest tested, which was 400 mg/kg, equal to 17 mg/kgbw per day, in rats and 7000 mg/kg, equal to 96 mg/kgbw per day, in mice.

Famoxadone was tested for genotoxicity in an adequate range of studies, both in vitro and in vivo. The results observed were largely negative. Although in one study famoxadone produced a weak clastogenic effect in vitro, the Meeting did not consider this to be toxicologically significant.

The Meeting concluded that famoxadone is unlikely to pose a genotoxic risk to humans.

Because the results of the studies of carcinogenicity were negative, the Meeting concluded that famoxadone is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproductive toxicity in rats, the NOAEL for adult rats and their offspring was 200 mg/kg, equal to 11.3 mg/kgbw per day in adults, on the basis of systemic toxicity in the parental rats and reduced body-weight gain in the offspring at a dose of 800 mg/kg, equal to 45 mg/kgbw per day; no other signs of reproductive toxicity were observed at this dose, the highest tested. In studies of developmental toxicity in rats and rabbits, no effects were observed in fetuses at doses of 1000 mg/kgbw per day, the highest dose tested. The results from the two studies of developmental toxicity and the study of reproductive toxicity did not reveal any increased susceptibility of fetuses or pups to famoxadone.

In 28-day studies of immunotoxicity in rats and mice, no evidence of immunotoxicity was found in rats receiving diets containing famoxadone at a concentration of 800 mg/kg, equal to 55 and 57 mg/kgbw per day in males and females respectively, or in mice receiving diets containing famoxadone at a concentration of 7000 mg/kg, equal to 1664 mg/kgbw per day in females, the highest doses tested. In male mice, there was a minimal but significant reduction in the primary humoral response to sheep erythrocytes at a dose of 7000 mg/kg; the NOAEL for this activity in male mice was thus 2000 mg/kg, equal to 327 mg/kgbw per day. The toxicological significance of this effect was considered to be minimal.

Clinical and microscopic evidence of lens opacities was clearly observed in female and male dogs (in both the 3-month and 1-year studies), at doses below those at which any other effects were observed in any other species. The mechanism by which these effects are induced is not understood.

Famoxadone does not appear to be neurotoxic. Some observations of minor effects made in an experiment investigating acute neurotoxicity were attributed to general malaise. Other than some clinical observations in males and females fed famoxadone at the highest dose in the 3-month study in dogs (myotonic twitching, possibly a result of high concentrations of serum potassium), no evidence for neurotoxicity was found in any other studies of toxicity, including a short-term study of neurotoxicity in rats.

The Meeting concluded that the existing database on famoxadone was adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

An ADI of 0–0.006 mg/kg bw was established for famoxadone based on the NOAEL of 1.2 mg/kg bw per day in a 1-year study in dogs treated by gavage, with a safety factor of 200; an extra safety factor was added because this study in dogs is not viewed as a long-term study. The critical effect was the occurrence of cataracts in dogs at 300 mg/kg, equal to 8.8 mg/kg bw per day; some of these cataracts developed late in the study, indicating that progression might have been possible, had a long-term study been conducted.

The Meeting established an acute reference dose (RfD) of 0.6 mg/kg bw for famoxadone on the basis of a NOAEL of 61.6 mg/kg bw per day, the only dose tested, in a study of haematotoxicity in rats treated for 16 days and a safety factor of 100.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of toxicity and carcinogenicity ^a	Toxicity	700 mg/kg, equal to 96 mg/kg bw per day	2000 mg/kg, equal to 274 mg/kg bw per day
		Carcinogenicity	7000 mg/kg, equal to 887 mg/kg bw per day ^c	—
Rat	2-year study of toxicity and carcinogenicity ^a	Toxicity	200 mg/kg, equal to 8.4 mg/kg bw per day	400 mg/kg, equal to 17 mg/kg bw per day
		Carcinogenicity	400 mg/kg, equal to 17 mg/kg bw per day ^c	—
	Two-generation study of reproductive toxicity ^a	Parental toxicity	200 mg/kg, equal to 11 mg/kg bw per day	800 mg/kg, equal to 45 mg/kg bw per day
		Offspring toxicity	200 mg/kg, equal to 11 mg/kg bw per day	800 mg/kg, equal to 45 mg/kg bw per day
	Study of developmental toxicity ^b	Maternal toxicity	250 mg/kg bw per day	500 mg/kg bw per day
		Offspring toxicity	1000 mg/kg bw per day ^c	—
	Special study of haematotoxicity ^a	Anaemia	800 mg/kg, equal to 62 mg/kg bw per day for 16 days	800 mg/kg, equal to 62 mg/kg bw per day for 30 days
	Single-dose study of neurotoxicity ^b	Neurotoxicity	2000 mg/kg bw ^c	—
Rabbit	Study of developmental toxicity ^b	Neurotoxicity	800 mg/kg, equal to 47 mg/kg bw per day ^c	—
		Maternal toxicity	1000 mg/kg bw per day ^c	—
		Offspring toxicity	1000 mg/kg bw per day ^c	—
Dog	1-year study of toxicity ^a	Toxicity	40 mg/kg, equal to 1.2 mg/kg bw per day	300 mg/kg, equal to 8.8 mg/kg bw per day

^a Diet
^b Gavage
^c Highest dose tested

Estimate of acceptable daily intake for humans

0–0.006 mg/kg bw

Estimate of acute reference dose

0.6 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

- Observations in humans
- Investigation of species differences in erythrocyte sensitivity to haemolysis
- Investigation of the mechanisms by which cataracts are formed in dogs

Summary of critical end-points for famoxadone

Absorption, distribution, excretion and metabolism

Rate and extent of oral absorption	About 40% absorbed and >75% of the administered dose eliminated in faeces in 24 h
Dermal absorption	No study of direct dermal absorption available
Distribution	Distributed throughout the body; tissue residues generally very low; highest concentrations in liver and fat
Potential for accumulation	Low, due to rapid excretion
Rate and extent of excretion	>75% excretion within 24 h
Metabolism in animals	Extensive
Toxicologically significant compounds (animals, plants and environment)	Parent

Acute toxicity

Rat, LD ₅₀ , oral	>5000 mg/kg bw
Rat, LD ₅₀ , dermal	No data
Rat, LC ₅₀ , inhalation	5.3 mg/l (4 h)
Rabbit, LD ₅₀ , dermal	>2000 mg/kg bw
Rabbit, dermal irritation	Mild irritant
Rabbit, ocular irritation	Mild irritant
Skin sensitization	Not sensitizing (Magnusson and Kligman)

Short-term studies of toxicity

Target/critical effect	Body-weight gain decrement, hepatotoxicity, regenerative haemolytic anaemia and lens opacities
Lowest relevant oral NOAEL	1.2 mg/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	250 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data available

Genotoxicity

No genotoxic potential

Long-term toxicity and carcinogenicity

Target/critical effect	Decreased body-weight gain, hepatotoxicity and regenerative haemolytic anaemia
Lowest relevant NOAEL	8.4 mg/kg bw per day: (2-year study in rats)

Carcinogenicity

No carcinogenic potential

Reproductive toxicity

Reproductive target/critical effect	Reduced parental and offspring body weight, clinical signs
Lowest relevant reproductive NOAEL	11 mg/kg bw per day
Developmental target/critical effect	Not teratogenic embryotoxic or fetotoxic
Lowest relevant developmental NOAEL	>1000 mg/kg bw per day (rats)

Neurotoxicity/delayed neurotoxicity

Target/critical effect	None
Lowest relevant NOAEL	>1000 mg/kg bw

90-day study of neurotoxicity

Target/critical effect	None
Lowest relevant NOAEL	>47 mg/kg bw per day

Other toxicological studies

None available

Medical data

None available

Summary	Value	Study	Safety factor
ADI	0–0.006 mg/kg bw	Dog, 1-year study, cataracts	200
Acute RfD	0.6 mg/kg bw	Rat, study of haematotoxicity in rats, haemolytic anaemia	100

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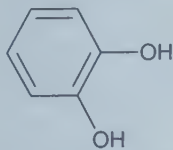
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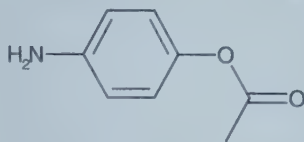
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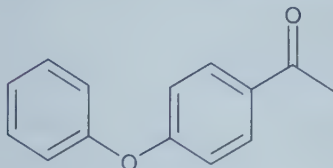
APPENDIX

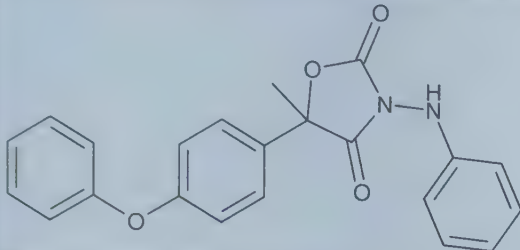
Famoxadone and its metabolites in rats and dogs

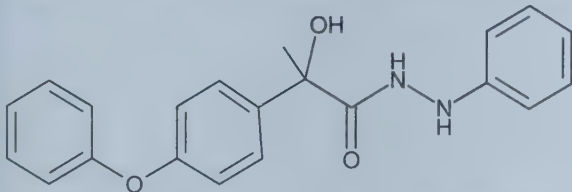
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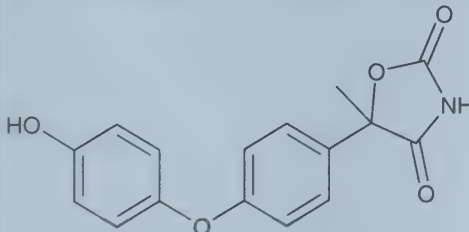
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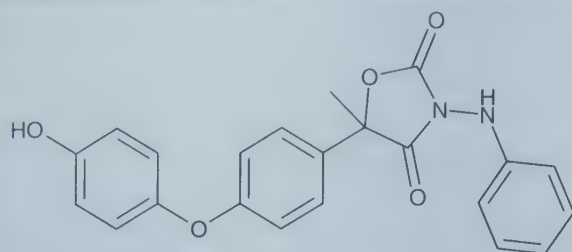
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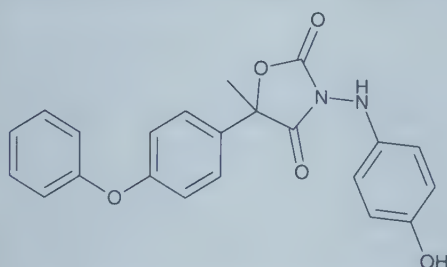
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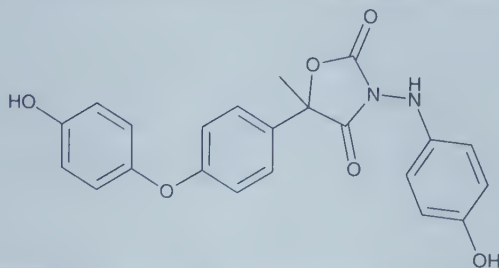
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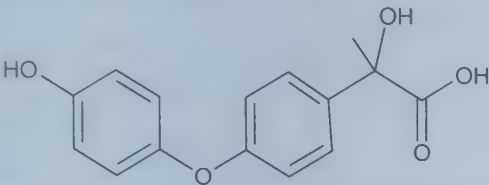
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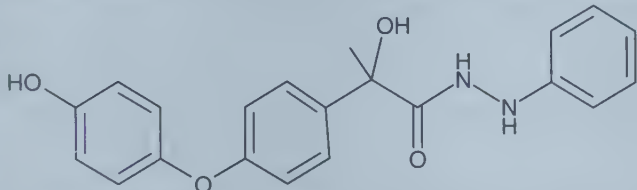
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CAS number:	NA	Relative molecular mass:	390.40	
Structural formula:	C ₂₂ H ₁₈ N ₂ O ₅	Observed in:	rats, dogs	

IN-KZ532	CAS name:	3-[(4-hydroxyphenyl)amino]-5-methyl-5-(4-phenoxyphenyl)-2,4-oxazolidinedione	
			
CAS number:	NA	Relative molecular mass:	390.40
Structural formula:	C ₂₂ H ₁₈ N ₂ O ₅	Observed in:	dogs

IN-KZ534	CAS name:	5-[4-(4-hydroxyphenoxy)phenyl]-3-[(4-hydroxyphenyl)amino]-5-methyl-2,4-oxazolidinedione	
			
CAS number:	NA	Relative molecular mass:	406.40
Structural formula:	C ₂₂ H ₁₈ N ₂ O ₆	Observed in:	rats, dogs

IN-ML436	CAS name:	alpha-hydroxy-4-(4-hydroxyphenoxy)-alpha-methyl-4-phenoxybenzeneaceticacid	
			
CAS number:	NA	Relative molecular mass:	274.28
Structural formula:	C ₁₅ H ₁₄ O ₅	Observed in:	rat

IN-ML815	CAS name:	alpha-hydroxy-4-(4-hydroxyphenoxy)-alpha-methylbenzeneacetic acid 2-phenylhydrazide	
			
CAS number:	NA	Relative molecular mass:	364.40
Structural formula:	C ₂₁ H ₂ ON ₂ O ₄	Observed in:	dogs

MALATHION (addendum)

*First draft prepared by
Timothy C. Marrs
Food Standards Agency, London, England*

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Explanation

Malathion was evaluated by the JMPR in 1963, 1965 and 1966 (Annex 1, references 2, 3 4, and 6). An acceptable daily intake (ADI) of 0–0.02 mg/kg bw was assigned at each Meeting. Malathion was re-evaluated by the JMPR in 1997 (Annex 1, references 80 and 81), when an ADI of 0–0.3 mg/kg bw was assigned. Malathion was re-evaluated at the present Meeting in order to establish an acute reference dose (RfD), at the request of the Codex Committee on Pesticide Residues. The Meeting reviewed a study in humans, some studies of toxicity in animals, and studies of genotoxicity, which had been produced since the last evaluation by the JMPR. The FAO and WHO were in the process of revising the specifications for malathion technical material.

Evaluation for acute reference dose

1. Effects on enzymes and other biochemical parameters

A study was undertaken by Fulcher et al. (2001) to investigate the effects of orally-administered malathion (purity, 96%) on cholinesterase activity in CrI:CD®BR rats. In this study, which was a supplement to a study of developmental neurotoxicity (see below, and Fulcher, 2003), pregnant rats, offspring at various stages of development before weaning, and young adults (aged 7–8 weeks) were given single or repeated doses of malathion of up to 450 mg/kg bw. Groups of nine mated female animals were given malathion at a daily dose of 0, 5, 50 or 150 mg/kg bw by gavage on days 6–20 of gestation. Eight rats per group were killed 3 h after the last dose, litter data were assessed and maternal and fetal plasma, erythrocyte and brain cholinesterase activities were determined. Additional groups of 10 mated females were treated with the same daily doses from day 6 of gestation until postnatal day 10. These dams were allowed to litter and rear their young until weaning on postnatal day 21, the litters being culled to four males and four females each on postnatal day 4, when measurements of plasma, erythrocyte and brain cholinesterase activity were made for selected culled offspring from each litter. The remaining offspring from eight litters per

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group were then given malathion during postnatal days 11–21, in order to assess effects on survival, body-weight gain and plasma, erythrocyte and brain cholinesterase activities; selected offspring were killed on postnatal day 21 or 60. Another group of eight untreated pregnant females was allowed to give birth to litters and to rear their young. The litters were culled to five pups of each sex on postnatal day 4. On postnatal day 11, one male and one female per litter were assigned to each group (eight pups of each sex per group) and given a single dose of malathion of 0, 5, 50, 150 or 450 mg/kg bw. These pups were killed 2 h after dosing, and plasma, erythrocyte and brain cholinesterase activities were determined. Additionally, young adult animals (eight of each sex; aged 7–8 weeks) were given malathion at a dose of 0, 5, 50, 150 and 450 mg/kg on one occasion, and were killed 2 h later and plasma, erythrocyte and brain cholinesterase activities were determined. Determination of plasma, erythrocyte and brain cholinesterase activities was carried out for eight male and eight female young adults that were given malathion at a dose of 0, 5, 50 or 150 mg/kg bw per day for 11 days and killed 2 h after the last dose. Determination of cholinesterase activity was undertaken using a modified Ellman method (Environmental Protection Agency, 1996). Because of the complicated study design, the findings are discussed for all groups (i.e. pregnant rats, offspring at various stages of development and young adults) by dose. In all cases, inhibition of cholinesterase activity is expressed as group mean reduction (%) relative to values for the appropriate concurrent control group.

450 mg/kg bw per day

Among offspring of untreated dams given a single dose of malathion of 450 mg/kg bw on postnatal day 11, body tremors were observed 1–2 h after dosing in five out of 16 animals and an additional pup was killed in extremis. Marked inhibition of plasma, erythrocyte and brain cholinesterase activities was seen, with inhibitions of 54%, 72% and 84% in plasma, erythrocytes and brains of males, respectively, and inhibitions of 52%, 61% and 81% in plasma, erythrocytes and brains of females, respectively. In young adults given a single dose of 450 mg/kg bw, plasma and erythrocyte cholinesterase activities were inhibited by 24% and 25% in males, respectively, and by 11% and 17% in females, respectively. Brain acetylcholinesterase activity was not inhibited in animals of either sex.

150 mg/kg bw

No adverse effects were seen in dams or young adult rats given repeated doses of malathion at 150 mg/kg bw per day, as assessed by clinical signs, body weight and body-weight gain, including during pregnancy and lactation and at necropsy. Litter data on day 20 of gestation and on postnatal day 11, fetal weight on day 20 of gestation, pup weight on postnatal day 1 and pup body-weight gain until postnatal day 11 were unaffected by treatment of the dams. In pups (taken from dams treated from day 6 of gestation until postnatal day 10) treated with malathion at a dose of 150 mg/kg bw from postnatal day 11 until weaning on postnatal day 21, no adverse effects were observed as assessed by clinical signs, survival or body-weight gain through to postnatal day 60. On day 20 of gestation, the dams treated with malathion exhibited depression of erythrocyte cholinesterase activity (inhibition of 51% compared with controls), but there was no inhibition of plasma or brain cholinesterase activity. Fetal plasma and erythrocyte cholinesterase activities were inhibited by 15% and 19%, respectively, on day 20 of gestation, while the activity of brain cholinesterase was unaffected. On postnatal day 4, cholinesterase activity in pups was comparable in test and control groups. Direct treatment of offspring of untreated dams was associated with marked inhibition of plasma, erythrocyte and brain cholinesterase activities on

postnatal day 11: plasma cholinesterase activity was inhibited by 36% in males and 35% in females; erythrocyte cholinesterase activity was inhibited by 55% in males and 48% in females; and brain cholinesterase activity was inhibited by 44% in males and 48% in females. For young adults, a single dose of 150mg/kgbw had no significant effect on cholinesterase activity in plasma, erythrocytes or brain. Direct treatment of the offspring of treated dams with 11 doses administered during postnatal days 11–21 caused inhibition of plasma, erythrocyte and brain cholinesterase activities; inhibition in erythrocytes was marked. Plasma cholinesterase activity was inhibited by 24% in males and 32% in females, erythrocyte cholinesterase activity was inhibited by 67% in males and 68% in females and brain cholinesterase activity was inhibited by 16% in both sexes. Young adult males treated for 11 days showed inhibition of plasma and erythrocyte cholinesterase activities of 13% and 43%, respectively, while brain cholinesterase activity was unaffected. Young female adults treated for 11 days showed inhibition of plasma and erythrocyte cholinesterase activities of 13% and 48%, respectively, while the activity of brain cholinesterase was not affected. On postnatal day 60 (39 days after the end of treatment), offspring showed no discernible treatment-related effect on the activity of erythrocyte or brain cholinesterase, but plasma cholinesterase activity was inhibited by 13% and 23% in males and females, respectively.

50mg/kgbw

No adverse effects were seen in dams or young adults rats given malathion at a dose of 50mg/kgbw per day, as assessed by clinical signs, body weight and body-weight gain, including during pregnancy and lactation and at necropsy. Litter data on day 20 of gestation and postnatal day 11, fetal weight on day 20 of gestation, pup weight on postnatal day 1 and pup body-weight gain until postnatal day 11 were unaffected by treatment of the dams. Treatment at this dose had no effect on pups from postnatal day 11 until weaning on postnatal day 21, as assessed by clinical signs, survival or body-weight gain through to postnatal day 60. On day 20 of gestation, the dams showed marginal inhibition of erythrocyte cholinesterase activity (19%), but neither plasma nor brain cholinesterase activities were inhibited. Fetal plasma and erythrocyte cholinesterase activities were marginally inhibited on day 20 of gestation (14% and 11% respectively), while brain cholinesterase activity was unaffected. On postnatal day 4, plasma, erythrocyte and brain cholinesterase activities in the pups were comparable in treated and control groups. At postnatal day 11, treatment of offspring of untreated dams was associated with inhibition of plasma and erythrocyte cholinesterase activities (inhibitions of 19% and 25% in males, and 16% and 23% in females, respectively), while brain cholinesterase activity was only slightly affected (inhibition of 6% and 10% in males and females, respectively). For young adults, treatment with a single dose of malathion had no significant effect on the activity of cholinesterase in plasma, erythrocytes or brain. Treatment of the offspring during postnatal days 11–21 caused inhibition of plasma and erythrocyte cholinesterase activities, inhibition being 19% and 39% in males and 19% and 34% in females, respectively. Cholinesterase activity in the brain was unaffected. Young adults treated with malathion for 11 days showed a minor degree of inhibition of plasma cholinesterase activity (11% in males and 15% in females), while erythrocyte cholinesterase activity was inhibited by 20% in both sexes. Activity of the brain enzyme was not inhibited. On postnatal day 60 (39 days after the end of treatment), there was no discernible effect on the erythrocyte or brain cholinesterase activity in offspring, while the plasma cholinesterase activity was inhibited by 5% and 16% in males and females, respectively.

5 mg/kg bw

At 5 mg/kg bw per day, there were no adverse effects in dams and young adult rats as assessed by clinical signs, body weight and body-weight gain, including during pregnancy and lactation and at necropsy. Litter data on day 20 of gestation and postnatal day 11, fetal weight on day 20 of gestation, pup body weight on postnatal day 1 and pup body-weight gain through to postnatal day 11 were not affected by treatment given to the dams. No effect of treatment of pups from postnatal day 11 to weaning on postnatal day 21 was observed in respect of clinical signs, survival or body-weight gain through to postnatal day 60. On day 20 of gestation, the dams did not show inhibition of erythrocyte or brain cholinesterase activity, although plasma cholinesterase activity was inhibited by 12%. Fetal plasma, erythrocyte and brain cholinesterase activities were unaffected by treatment. On postnatal day 4, cholinesterase activity in pups was comparable in the test groups and the controls. Treatment of offspring of untreated dams on postnatal day 11 did not affect plasma, or brain cholinesterase activities, but erythrocyte cholinesterase activity was observed to be marginally (16%) inhibited. For young adults, treatment with a single dose of malathion had no significant effect on cholinesterase activity in plasma, erythrocytes or brain. Treatment of the offspring during postnatal days 11–21 produced only minor degrees of inhibition of plasma (13% and 10% in males and females, respectively) and erythrocyte (17% and 15% in males and females, respectively) cholinesterase activity. The activity of brain cholinesterase was unaffected. Young adults treated for 11 days showed no inhibition of plasma, erythrocytic or brain cholinesterase activity. On postnatal day 60 (39 days after the end of treatment), offspring showed no discernible effect on cholinesterase activity, except for plasma cholinesterase in females, which was inhibited by 18%. The no-observed-adverse-effect level (NOAEL) for the study was 50mg/kgbw. Inhibition of brain cholinesterase activity was observed in offspring of untreated females after direct treatment with a single dose of malathion of 150mg/kg bw on postnatal day 11. In this study, erythrocyte cholinesterase activity was more sensitive to inhibition induced by malathion than was brain cholinesterase activity (Fulcher et al., 2001).

2. Toxicological studies

2.1 Genotoxicity

Table 1 summarizes the results of studies of genotoxicity with malathion.

Table 1. Results of studies of genotoxicity with malathion

End-point	Test object	Concentration/dose	Purity	Results	Reference
<i>In vitro</i>					
Chromosomal aberration	Human lymphocytes	12.5–800 µg/ml –S9, 75–1800 µg/ml +S9	96%	Positive –S9 (at toxic concentrations)	Edwards (2001a) ^a
Gene mutation	Mouse lymphoma cells (L5178Y)	125–2000 µg/ml –S9, 250–2200 µg/ml –S9	96%	Positive ± S9 (at markedly toxic concentrations)	Edwards (2001b) ^b
<i>In vivo</i>					
Unscheduled DNA synthesis	Male Wistar rats	500–2000 mg/kg bw (by gavage)	96%	Negative	Meerts & van de Waart (2003) ^c

S9, 9000 × g supernatant fraction from rodent liver
^aTest complied with GLP (United States Food and Drug Administration, 21CFR58; Japanese Ministry of Health and Welfare, PAB 414; European Commission, 1999/11/EC) and with OECD Guideline 473
^bTest complied with GLP (United Kingdom SI 3106; OECD ENV/MC/CHEM(98)17; and European Commission 1999/11/EC)
^cTest complied with GLP (United States Food and Drug Administration, 21CFR58; United States Environmental Protection Agency, 40CFR160, 40CFR792) and with OECD Guideline 486

2.2 *Special studies*

(a) *Teratogenicity*

A study of teratogenicity in rabbits, evaluated by the 1997 JMPR, was recently re-evaluated by Robinson (2002), and is briefly described again here. Subsequent to a range-finding study, malathion (purity, 92.4%) in corn oil was administered at a dose of 25, 50 and 100 mg/kg bw per day by gavage to groups of 20 mated New Zealand white female rabbits during days 6–18 of gestation; controls received corn oil only. Although there were no statistically significant differences in survival between the treated and control groups, no deaths occurred among the controls, while there were four deaths in the group receiving the lowest dose, three in the group receiving the intermediate dose and two in the group receiving the highest dose. Both instances of mortality in the group receiving the highest dose resulted from accidental intrapulmonary intubation during dosing. No clear cause of death was established for mortality occurring at the two lower doses. The study authors concluded that there was no dose–response relationship with regard to mortality. There was a decrease in maternal body-weight gain during days 6–18 of gestation at a dose of 50 and 100 mg/kg bw per day. On days 12, 18 and 29, the mean body weight of the group receiving the highest dose was lower than that of the controls. Therefore, the NOAEL for maternal toxicity was 25 mg/kg bw per day. There was a slight increase in the mean number and per cent of resorptions at ≥ 50 mg/kg bw per day. There was no difference in fertility, number of corpora lutea, implantation sites, litter size or fetal weight and length. No other signs of toxicity were seen in does or fetuses, nor was there any evidence for teratogenicity. The 1997 Meeting considered that the NOAEL was 25 mg/kg bw per day for maternal toxicity, on the basis of decreased body-weight gain at the intermediate dose, and 100 mg/kg bw per day for fetal toxicity, on the basis of the failure to observe fetal toxicity at any dose (Siglin, 1985). The embryoletality observed in this study, and in the range-finding study was re-evaluated by Robinson (2002). In the re-evaluation, the data for animals treated at a dose of 25, 50 or 100 mg/kg bw per day were combined from these two studies; it was concluded that treatment with malathion had no effect on postimplantation loss (or any other parameter for development). Thus, the NOAEL of 100 mg/kg bw per day for fetal toxicity was maintained.

(b) *Developmental neurotoxicity*

A study of developmental neurotoxicity with orally administered malathion (purity, 96%) in Crl:CD®BR rats was undertaken by Fulcher et al. (2002) in compliance with GLP (United Kingdom SI 3106, OECD, ENV/MC/CHEM(98)17, European Commission, 1999/11/EC) and with United States Environmental Protection Agency (EPA) guideline subdivision F, OPPTS 870.6300.

Malathion was administered to groups of at least 21 mated females on day 6 of gestation until postnatal day 10, and to their offspring on postnatal days 11–21 at a dose of 5, 50 or 150 mg/kg bw per day; a control group received the vehicle, namely corn oil. The F₀ dams were inspected twice daily. Body weights were recorded on days 0, 3, 6, 10, 14, 17 and 20 of gestation and thereafter daily until parturition. The dams were also weighed on postnatal days 1, 4, 11, 14, 17 and 21. Food consumption was recorded for the following periods: days 0–2, 3–5, 6–9, 10–13, 14–16 and 17–19 of gestation and postnatal days 1–3, 4–6, 7–10, 11–13, 14–16 and 17–20. Behavioural assessments were performed on at least 10 dams per group. These comprised arena and in-hand observations on days 12 and 18 of gestation and postnatal days 4 and 10. Arena observations comprised degree of eyelid

closure, posture, gait, tremor, twitches and convulsions or absence thereof, activity counts, rearing counts, grooming, urination and defecation. In-hand observations comprised ease of removal from cage, salivation, lacrimation, piloerection, exophthalmos, pupil closure reflex, condition of fur and reactivity to handling. All offspring were examined 24h after birth; the number of live and dead offspring and their body weights and sex were recorded, and they were observed clinically. A daily record was maintained of mortality in pups from birth until postnatal day 21. On postnatal day 4, the litters were culled to eight offspring per litter; offspring from each litter were allocated for behavioural assessment. Offspring were weighed on postnatal days 1, 4, 7, 11–21 and 28, and weekly thereafter. On postnatal day 11, the litters were culled to seven animals per litter to provide a male or female pup for necropsy, while on postnatal day 21 litters were culled to six animals, to provide one additional pup per litter for necropsy. If possible, this pup was of the opposite sex to that culled on postnatal day 11. Pups were inspected twice daily for signs of ill health. Behavioural observations on the pups were carried out as follows: arena observations were carried out on postnatal days 4, 11, 21, 35, 45 and 60, in-hand observations on postnatal days 35, 45 and 60, assessment of motor activity on postnatal days 13, 17, 22 and 59, assessment of auditory startle response inhibition on postnatal days 23/24 and 60/61, assessment of auditory startle pre-pulse inhibition on postnatal days 23/24 and 60/61, and assessment of learning and memory (Morris maze) on postnatal days 23/24 and 61/62. Sexual maturation was ascertained by examination for balano-preputial separation in males from postnatal day 38 onwards and by examination for vaginal opening in females from postnatal day 28 onwards. On postnatal day 11, one pup per litter was killed and 10 pups of each sex were perfused for neuropathological examination. Dams and one pup per litter were killed after weaning at postnatal day 21, with 10 male and 10 female pups being perfused with fixative for neuropathological examination. The remainder of the offspring were killed on postnatal days 63–67 and 10 males and 10 females were perfused with fixative for neuropathological examination of the brain. Additionally, selected organ weights were recorded for one male and one female per litter. Treatment of the dams at any dose had no effect on survival, clinical condition, body-weight gain or food intake, either during gestation or lactation. No effects related to treatment with the test material were observed on length of gestation, and parturition was normal; moreover, there were no effects on the results of behavioural assessments made on day 12 or 18 of gestation or postnatal day 4 or 10. Salivation after dosing was seen at all doses, including the controls. This effect, which was most severe at the highest dose, was considered to be a consequence of distaste for the formulation. Absolute and relative weights of reproductive organs and the brain did not differ between groups. Implantation counts, litter size on postnatal day 1 and survival to weaning (postnatal day 21) were similar in all groups. Body-weight gain of offspring in all groups was similar. In the offspring, most behavioural examinations showed little of note. During direct treatment of the offspring (postnatal days 11–21), tremors and underactivity were seen in four offspring from one litter, at 150 mg/kgbw; the effect was seen on days 7, 8 and 9 of treatment. This was thought to be a direct effect of treatment and not evidence for developmental neurotoxicity. On postnatal day 11, however, five female pups at 150 mg/kgbw per day failed to show an immediate surface righting reflex (only one pup had a slow surface righting reflex in the control group). At subsequent times (on postnatal day 22 in male pups and on postnatal day 59 in pups of both sexes), motor activity was not affected by treatment. On postnatal day 22, rearing and cage-floor activity was lower in treated females than in the controls, some differences being significant; however, this was not corroborated by an increase in time to complete the Morris water maze. Also there was considerable variation within the groups. On postnatal day 23/24, group mean startle amplitudes for treated offspring during startle habituation and during pre-pulse inhibition were higher than those of controls, and some of these

differences were significant. However, no dose–response relationship was observed. No such findings were elicited on postnatal day 61/62. At examination of the dams (postnatal day 21) and the pups (postnatal days 11, 21, 63–67) post mortem, no histopathological changes of significance were found. There were no effects of the treatment on the weights of the reproductive organs of dams (postnatal day 21) or offspring (postnatal day 63–67). There were no effects on brain weight, length and width among the pups on postnatal days 11, 21 and 63–67. Neuropathological examination of the offspring on postnatal day 21 and 63–67 did not reveal any findings that were related to treatment. The study authors considered that the NOAEL for the study was 50 mg/kg bw per day for developmental neurotoxicity (slower surface righting reflex at the highest dose of 150 mg/kg bw per day) and 150 mg/kg bw per day for maternal toxicity. The present reviewer agreed with this assessment and noted that the effects observed were likely to be caused by current treatment rather than any permanent developmental neurotoxic effect.

3. Observations in humans

A randomized double-blind placebo-controlled ascending single dose study was carried out in humans treated orally with malathion; the study complied with good clinical practice (GCP) (CPMP/ICH/135/95) and GLP (OECD, USEPA 40 CFR 160). Forty-eight healthy men and women (aged 18–50 years) were given gelatin capsules containing malathion (purity, 95.8%) at a dose of 0.5, 1.5, 5, 10 or 15 mg/kg bw, while controls received a placebo comprising gelatin capsules containing lactose. A total of 48 subjects (38 men and 10 women) completed the study, which was divided into seven sessions. In the first session, three men were given malathion at a dose of 0.5 mg/kg bw. In the second session, another three men were given malathion at a dose of 1.5 mg/kg bw. Subsequently, seven men were given malathion at a dose of 5.0 mg/kg bw. Three and four men received malathion at a dose of 10 mg/kg bw in two separate sessions. There were three sessions in which three males, four males and seven females all received malathion at a dose of 15 mg/kg bw. In each session, one or more subjects received a placebo, which contained lactose (see Table 2 for experimental design). The subjects were kept under close observation from before dosing until 72 h after dosing. Any symptoms or clinical signs were recorded and blood pressure, pulse rate, respiratory rate and body temperature were determined the day before dosing, immediately before dosing and 2, 4, 8 and 24 h after dosing. Twelve-lead electrocardiograms (ECGs) were carried out 30 min before dosing and 2, 4, 8 and 24 h after dosing, and single channel continuous ECG was performed from 30 min before dosing until 4 h after dosing. Blood was collected for measurement of haematological and clinical chemical parameters at screening before entry into the study, before dosing and 24 h after dosing. Urine

Table 2. Design of a study in humans treated with a single dose of malathion

Session	No. of subjects ^a	Dose (mg/kg bw)					
		0 (placebo)	0.5	1.5	5.0	10.0	15.0
1	4 men	1	3	—	—	—	—
2	4 men	1	—	3	—	—	—
3	10 men	3	—	—	7	—	—
4	4 men	1	—	—	—	3	—
5	9 men	2	—	—	—	4	3
6	7 men	3	—	—	—	—	4
7	10 women	3	—	—	—	—	7

From Gillies & Dickson (2000)

analysis was performed at screening and 24 h after dosing. Blood for measurement of plasma and erythrocyte cholinesterase activity was taken at screening and on days -9, -7, -5, -2 and -1 and at -30 min (before dosing). Samples were also taken at 1, 2, 4, 8, 12, 24 and 48 h after dosing and on days 4, 7 and 14 after dosing. After separation into erythrocytes and plasma, samples were stored at -70°C . A modified Ellman method was used to assay cholinesterase activity, and these results were compared with those for placebo controls. Blood samples for the estimation of concentrations of malathion and malaoxon were taken before dosing and 1, 2, 4, 8 and 12 h and 1, 2 and 3 days after dosing. Concentrations of malathion and malaoxon were measured in the plasma of subjects receiving the highest dose (the limit of quantification of the analytical method for both malathion and its oxon was approximately 100 ng/ml). Urine was collected over the following periods: from 12 h before dosing until dosing; for 12 h after dosing; from 12–24 h after dosing; and from 24–48 h after dosing. These samples were analysed for malathion monocarboxylic acid, malathion dicarboxylic acid, dimethyl phosphate, dimethyl thiophosphate and dimethyl dithiophosphate. Criteria for withdrawal from the study were: (i) for individual subjects, $>25\%$ inhibition of plasma or erythrocyte cholinesterase activity compared with baseline activity at two consecutive time-points; (ii) for cohorts, $>15\%$ inhibition of plasma or erythrocyte cholinesterase activity at two consecutive time-points.

No test-material-related clinical changes were seen and ECGs, haematology and clinical chemistry were unaffected by the malathion. No significant changes in plasma or erythrocyte cholinesterase activity were observed when compared with activities before dosing or activities measured for placebo controls at any dose. Plasma concentrations of malathion and malaoxon in subjects receiving the highest dose (15 mg/kg bw) were below the limit of quantification; for this reason, samples from subjects receiving lower doses of malathion were not analysed. As no test material-related effect was observed during the study, the NOAEL was considered to be 15 mg/kg bw. This study complied with GCP (CPMP/ICH/135/95) and GLP (OECD, United States EPA, 40 CFR 160) (Gillies & Dickson, 2000).

Comments

As a supplement to a study of developmental neurotoxicity, a study was undertaken on the effects of orally administered malathion on the activity of cholinesterase. In this study, single or repeated doses of malathion of up to 450 mg/kg bw were administered orally to pregnant rats, pre-weaning offspring at various stages of development, and young adults. The NOAEL for the study was 50 mg/kg bw. Inhibition of brain cholinesterase activity was observed in offspring of untreated females who were given a single direct dose of 150 mg/kg bw on postnatal day 11.

A study of developmental toxicity in rabbits was evaluated by the 1997 JMPR. This study was re-evaluated in 2002. Malathion was administered at a dose of 25, 50 or 100 mg/kg bw per day by gavage to groups of mated female rabbits during days 6–18 of gestation. The NOAEL for maternal toxicity was 25 mg/kg bw per day on the basis of decreased maternal body-weight gain during dosing. There was no difference in fertility, number of corpora lutea, implantation sites, litter size or fetal weight and length. The NOAEL was 100 mg/kg bw per day for fetal toxicity on the basis of the absence of developmental toxicity at any dose.

In a study of developmental neurotoxicity, malathion was administered orally to groups of mated female rats from day 6 of gestation to postnatal day 10 and to their offspring from postnatal day 11 to postnatal day 21 at doses of 5, 50 or 150 mg/kg bw per day. Behavioural assessments were performed on both dams and pups, in the latter at intervals up to postnatal day 60. The NOAEL for the study was 50 mg/kg bw per day for developmental neurotoxicity (slower surface righting reflex at the highest dose of 150 mg/kg bw per day on postnatal day 11, but not subsequently) and 150 mg/kg bw per day for maternal toxicity. It was considered that the effects observed were likely to have been caused by current treatment rather than any permanent developmental neurotoxic effect, because neurotoxicity was not observed in the offspring at later time-points in the study.

The results of tests for chromosomal aberrations in human lymphocytes and gene mutation in mouse lymphoma cells were positive at cytotoxic concentrations. A test for unscheduled DNA synthesis in vivo in male rats gave negative results. This is consistent with the conclusions of the 1997 JMPR, which recorded that although the results of some tests in vitro on malathion were positive, malathion was not genotoxic in vivo.

An acceptable¹ randomized double-blind placebo-controlled ascending single oral dose study was carried out in healthy men and women aged 18–50 years. Malathion was administered in gelatin capsules at a dose of 0.5, 1.5, 5, 10 or 15 mg/kg bw. No test-material-related clinical changes were seen, nor were ECGs, haematology or clinical chemistry parameters affected by treatment with malathion. No significant changes in plasma or erythrocyte cholinesterase activities were observed when compared with activities before dosing or placebo controls at any dose. As no test-material-related effects were observed during the study, the NOAEL was considered to be 15 mg/kg bw.

After considering the new data made available to the Meeting and also the previous monograph, the Meeting established an acute RfD of 2 mg/kg bw on the basis of the study in humans and a safety factor of 10. It should be noted that this acute RfD is likely to be conservative as erythrocyte cholinesterase was more sensitive to inhibition by malathion than brain cholinesterase in the studies available to the Meeting. The Meeting considered that the use of data from studies in which pre-weaning pups received bolus doses of pesticides by direct dosing, particularly when they were also receiving the pesticide in unknown amounts from the dams via their milk, was inappropriate for the establishment of an acute RfD.

Estimate of acute RfD

2 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

Further observations in humans

¹ Annex 1, references 83, page 5.

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METHOXYFENOZIDE

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Explanation

Methoxyfenozide (*N*-*tert*-butyl-*N'*-(3-methoxy-*o*-toluoyl)-3,5-xylohydrazide) is a diacylhydrazine insecticide that acts as an ecdysone agonist. Methoxyfenozide has not been evaluated previously by the JMPR. The Meeting noted that the purity of the material tested (>98%) was higher than that of the material proposed for commercialization (97%), but concluded that the findings were applicable to the proposed technical specification and production material.

Evaluation for acceptable daily intake

The studies of toxicity with methoxyfenozide were performed between 1994 and 1998. The purity of the methoxyfenozide used in the majority of studies (98–99.2%) was higher than that of the proposed technical specification (purity, 97%). Data supplied confirmed that the specifications of the main toxicity batch were consistent with those of the production material. Overall, the Meeting concluded that the material tested was adequately representative of the production material.

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The toxicokinetics and metabolism of methoxyfenozide in rats were investigated using the following ^{14}C -labelled test materials: (i) [methoxyphenyl ring uniform label- ^{14}C]methoxyfenozide (“A-ring label”), radiochemical purity, 98.2%; (ii) *S*[dimethylphenyl ring uniform label- ^{14}C]methoxyfenozide (“B-ring label”), radiochemical purity, 99.0%; (iii) *t*-butyl [^{14}C]methoxyfenozide (“*t* label”), radiochemical purity, 96.4%.

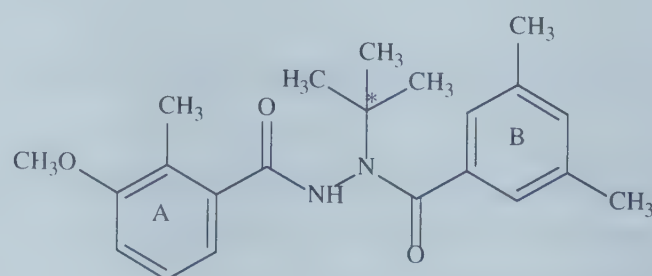
Rats

In a study of absorption, distribution, metabolism, and excretion that was designed to comply with the pesticide assessment guidelines of the United States Environmental Protection Agency (EPA) and Japan and that complied with good laboratory practice (GLP), forty-two groups of Sprague-Dawley rats (up to five animals of each sex per group) were given either A-ring, B-ring or *t*-butyl-labelled [^{14}C]methoxyfenozide at a nominal dose of 10 or 1000 mg of active substance/kg bw, by oral gavage. The test materials were prepared as suspensions in 0.5% aqueous methylcellulose. The [^{14}C]methoxyfenozide was combined with appropriate amounts of unlabelled (^{12}C) methoxyfenozide for dilution of radioactivity at the highest dose and with ^{13}C -labelled (A-ring carbonyl; B-ring methyl; *t*-butyl carbon) methoxyfenozide for identification of metabolites.

Three basic experimental designs were used: one to determine the excretion, distribution, and mass balance of radioactivity (120 h after dosing), one to evaluate the pharmacokinetics of radioactivity in the blood (to determine the time to C_{\max} and $\frac{1}{2} C_{\max}$), and one to determine the tissue distribution of radioactivity at C_{\max} , $\frac{1}{2} C_{\max}$ and 5 days after dosing. Biliary excretion was investigated in bile-duct cannulated rats given a dose of 10 mg/kg bw by gavage. In addition to the single dose experiments, one group of animals received diets containing unlabelled methoxyfenozide at a concentration of 200 mg/kg (equivalent to 20 mg/kg bw per day) for 2 weeks before receiving a single oral dose of [^{14}C -A-ring]methoxyfenozide at 10 mg/kg. Another group received five daily oral doses of [^{14}C -A-ring]methoxyfenozide at 10 mg/kg by gavage. The amount of radiolabel contained in exhaled air was determined using activated charcoal and sodium hydroxide traps and liquid scintillation counting (LSC) after appropriate sample preparation.

The excretion of radiolabel after a single oral dose of A-ring, B-ring or *t*-butyl-labelled methoxyfenozide followed a similar pattern, regardless of dose (representative data for A-ring-labelled material are presented in Tables 1–3). For each dose, the results for the

Figure 1. Structure of methoxyfenozide



* = *t*-butyl; A = A-ring; B = B-ring

Table 1. Recovery of A-ring-labelled [¹⁴C]methoxyfenozide (%) in rats

Dose (mg/kg bw)	Route	Sex	Time of sacrifice	Recovery of the administered dose (%)					
				Urine	Faeces	Bile	Tissues	Carcass	Total
1000	Oral	Male	Day 7	4.89	89.21	—	—	0.10	94.20
1000	Oral	Female	Day 7	7.50	87.00	—	—	0.05	94.55
1000	Oral	Male	Day 5	5.33	93.49	—	0.07	0.09	98.98
1000	Oral	Female	Day 5	8.92	89.81	—	0.01	0.10	98.85
10	Oral	Male	Day 5	7.06	96.81	—	0.08	0.06	104.01
10	Oral	Female	Day 5	11.95	92.32	—	0.01	0.10	104.38
10	Oral (bile)	Male	72 h	4.98	26.16	64	0.08	0.27	95.89
10	Oral (bile)	Female	72 h	22.15	35.04	38	0.12	1.34	97.38
10 ^a	Oral + gavage ^a	Male	Day 5	7.62	90.92	—	0.16	0.07	98.76
10 ^a	Oral + gavage ^a	Female	Day 5	12.17	90.01	—	0.01	0.06	102.26
10	Oral ^b	Male	15 min	5.00	71.54	—	3.30	2.07	97.75
10	Oral ^b	Female	15 min	8.47	66.30	—	3.46	3.24	95.44

From Watts & Longacre, 1998

^a 14 days at 200 mg/kg [¹²C]methoxyfenozide plus a single dose of [¹⁴C-A-ring]methoxyfenozide at 10 mg/kg bw administered by gavage

^b Six daily doses administered by gavage

Table 2. Toxicokinetic half-lives ($T_{1/2}$) for elimination and peak concentration of radiolabel in plasma in rats given a single oral dose of A-ring-labelled [¹⁴C]methoxyfenozide by gavage

Dose (mg/kg bw)	Sex	Plasma elimination $T_{1/2}$ (h)	α -Phase half-life (h)	β -Phase half-life (h)	Peak concentration (μ g equivalent/g)
1000	Male	9.4	0.2	24.2	27.68
1000	Female	10.1	0.5	22.5	29.74
10	Male	5.0	0.5	26.4	0.81
10	Female	4.6	0.2	19.6	0.59

From Watts & Longacre (1998)

Table 3. Mean concentration (pg equivalents/g) of radioactivity in blood, carcass, and tissues in rats given a single oral dose of A-ring-labelled [¹⁴C]methoxyfenozide

Tissue	C_{\max} (0.25 h) (10 mg/kg bw)		1/2 C_{\max} (2.0 h) (10 mg/kg bw)		5 days (1000 mg/kg bw)	
	Male	Female	Male	Female	Male	Female
Adrenals	32.38	51.56	32.56	18.58	0.8300	0.6631
Blood	12.61	37.84	16.39	16.71	0.4789	0.3530
Bone marrow	6.24	14.48	6.84	5.66	0.6104	1.4821
Brain	0.88	2.64	1.25	1.34	0.0936	0.0404
Carcass (residual)	8.53	34.61	8.97	30.10	0.9230	1.1200
Fat	5.30	13.22	10.38	12.31	0.4863	0.7756
Heart	9.89	21.96	12.23	10.50	0.2800	0.1553
Intestinal tract	791.70	1294.08	1493.84	1985.52	0.7872	1.3753
Kidneys	33.77	61.40	39.28	30.61	1.3949	0.9565
Liver	368.12	927.28	271.26	154.92	16.5151	1.8051
Lungs	16.92	33.45	16.84	14.78	0.3483	0.1300
Muscle (thigh)	6.23	11.61	6.38	6.33	0.1326	0.0605
Ovaries	—	40.20	—	20.07	—	0.2964
Plasma	17.34	46.38	25.23	24.56	0.0992	0.0402
Spleen	55.26	45.36	15.00	11.96	0.3702	0.3895
Stomach	6051.39	3538.61	1547.58	4129.65	0.3644	0.1960
Testes	1.55	—	4.89	—	0.0405	—
Thyroids	20.81	19.51	13.62	7.78	4.2711	2.5416

From Watts & Longacre (1998)

excretion, pharmacokinetics and tissue distribution of radioactivity were generally comparable for males and females. The overall recovery of ^{14}C ranged from 97% to 104% of the administered dose for all experiments, including the biliary excretion experiment. Most of the radiolabel was excreted during the first 24 h after administration of a single oral dose, primarily in the faeces, which contained 58–77% of the administered dose at day 1 (86–97% after 5–7 days). Females consistently excreted a greater proportion of the administered dose in the urine. Minimal amounts of radiolabel (0.10–0.23%) remained in the tissues and carcass at 5 days after a single dose. Less than 0.2% of the administered dose was exhaled as $^{14}\text{CO}_2$. These data indicate that the A-ring and B-ring remain associated during the metabolism of methoxyfenozide. Pre-treatment of animals with diets containing methoxyfenozide for 2 weeks, or with five daily doses of ^{14}C -labelled methoxyfenozide did not appreciably alter absorption or distribution (Table 1).

The biliary excretion of radiolabel after a single oral dose of [^{14}C -A-ring]methoxyfenozide at 10 mg/kg was investigated in bile-duct cannulated rats (Table 1). Biliary excretion was rapid, with 22% (females) and 50% (males) of the administered dose being excreted within 12 h. Overall, 38% (females) and 64% (males) of the radiolabel was excreted in bile within 72 h. Considerable variability between individual animals was seen in cannulated female rats (bile, 13–55%; and urine, 5–43% within 72 h), but the overall amount absorbed (in bile, urine, carcass and tissues) was similar for all four females (56–67%). Taking the biliary component into account, the overall extent of oral absorption of methoxyfenozide at a dose of 10 mg/kg bw was 60–70% in both sexes.

Toxicokinetic parameters for plasma and blood were investigated after a single dose of [^{14}C]methoxyfenozide (at a lower and higher dose for each labelling position) by gavage. Findings were similar, irrespective of labelling position. [^{14}C]Methoxyfenozide was rapidly absorbed, with maximum concentrations of radioactivity in blood and plasma being observed 15–30 min after dosing (Table 2). The maximum concentrations were similar for each radiolabel and sex at each dose, but tended to be slightly higher in males, especially at the lower dose. Peak blood concentrations were not proportional to the dose administered, i.e. peak concentrations in blood and plasma at the highest dose were only 24–68-fold higher than at the lowest dose, which is indicative of saturation of absorption. Values for the area under the curve (AUC) were not calculated. Elimination of radiolabel from the plasma followed a biphasic pattern (Table 2), with secondary peaks indicative of entero-hepatic circulation (especially in females). In rats given the test compound at the higher dose, the overall elimination half-life of radiolabel from the plasma was longer than in rats given the lower dose. These results are consistent with saturation of the elimination pathway for parent compound and/or its metabolites in rats at the higher dose in both sexes, with these effects being more prominent in females.

The tissue distribution of radioactivity was investigated after a single dose of A-ring-labelled or *t*-labelled methoxyfenozide administered by gavage (at C_{max} , $\frac{1}{2} C_{\text{max}}$ and at 5 days after dosing at 10 or 1000 mg/kg bw), and after a single dose of B-ring-labelled methoxyfenozide (5 days after dosing at 10 mg/kg bw). Tissue distribution was also investigated after dosing with A-ring-labelled methoxyfenozide at 10 mg/kg bw as a pulse dose (5 days after dosing) and as a repeated dose (at 0.25 h after the last dose, at about C_{max}).

Similar results were seen in all experiments. The absorbed radioactivity was widely distributed, with the highest concentration of absorbed radioactivity found in the liver at 0.5–2 h after dosing (the higher concentrations found in the stomach and intestinal tract

were attributed to largely unabsorbed material). Tissue distributions for the A-ring radiolabel are given in Table 3. Clearance from the body was extensive; 5 days after a single dose of 10 mg/kg bw, the highest percentage of radioactivity, representing <0.1% of the administered dose, was found in the liver (Watts & Longacre, 1998).

1.2 Biotransformation

A proposed metabolic pathway for methoxyfenozide in rats is shown in Figure 2.

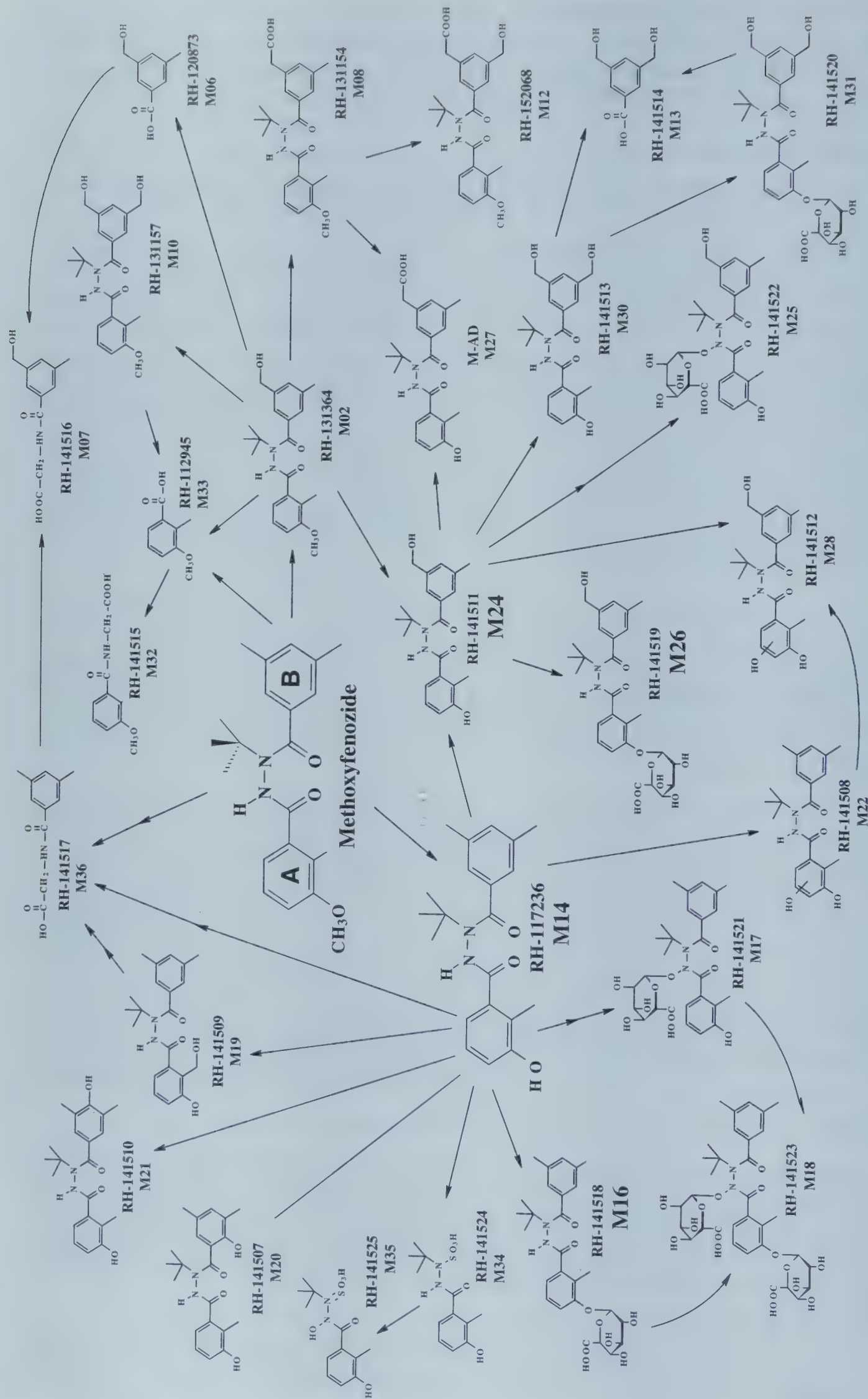
Metabolites in the urine, faecal, and bile samples from the study described above were identified and quantified. Most data were obtained for methoxyfenozide labelled with ^{14}C in the A ring and *t*-butyl group. Urine samples (mostly collected up to 24 h after dosing) were analysed directly by reverse-phase high-performance liquid chromatography (HPLC) and normal-phase thin-layer chromatography (TLC). Faeces (collected up to 24–48 h after dosing) were homogenized, extracted with acetonitrile and then partitioned to yield methylene chloride and aqueous fractions (which were analysed by reverse-phase HPLC and TLC) and post-extraction solids. Bile samples were analysed directly by reverse-phase HPLC and liquid chromatography–mass spectrometry (LC–MS). Metabolites were characterized and identified by TLC, gas chromatography–mass spectrometry (GC–MS) and/or LC–MS and compared with available reference standards. Selected metabolites were also derivatized for structural confirmation.

[^{14}C]Methoxyfenozide was metabolized extensively. A total of 31 metabolites, of which 25 were identified, were isolated from the rat urine and faeces. In addition, 24 metabolites were found in the bile and characterized; of these, 12 were identified (four were unique to bile). Table 4 summarizes the distribution of metabolites in the urine and faeces for methoxyfenozide labelled on the A-ring. Similar patterns were seen with B-ring, and *t*-butyl-labelled methoxyfenozide.

Parent compound was found only in the faeces (not in the urine or bile) and comprised 14–26% and 30–39% of the administered dose for animals at the lower and higher doses, respectively, indicating that animals at the lower dose metabolized a greater fraction of the administered dose compared with animals at the higher dose. Seven metabolites (M10, M14, M16, M22, M24, M28, M30) were found to be present in faeces plus urine each at >2% of the administered dose. The predominant metabolites were M14 (desmethylated parent) and M24 (hydroxy methyl derivative). Parent compound plus these seven metabolites accounted for 74–90% of the administered dose (in faeces plus urine) in all groups. For each of these groups, the total of parent plus identified metabolites accounted for ≥83% of the administered dose, i.e. the metabolic profile of methoxyfenozide in faeces and urine was well defined. Less than 5% of the administered dose was present as metabolites formed from the cleavage of the amide bridge.

Two metabolites, M16 (A-ring glucuronide of M14) and M26 (A-ring glucuronide of M24), were the main metabolites in bile. M16 was present at 13% and 18% in males and females respectively, M26 was present at 5% in males and 11% in females, all other metabolites represented <3% of the administered dose. The presence of M16 and M26 at higher concentrations in bile than in faeces indicates that these two metabolites were subject to subsequent hydrolysis.

Figure 2. Proposed metabolic pathway for methoxyfenozone in rats



From Watts & Longacre (1998)

M14 and M24 = main faecal metabolites

M16 and M26 (glucuronides of M14 and M24) = main biliary metabolites

Table 4. Major metabolites of A-ring-labelled methoxyfenozide in rats (% of administered dose)

Metabolite	Male			Female		
	Faeces	Urine	Subtotal	Faeces	Urine	Subtotal
<i>1000 mg/kg bw</i>						
Active substance	39.34	—	39.34	36.13	—	36.13
M14	14.59	0.46	15.05	15.04	2.92	17.96
M22	2.95	—	2.95	1.43	—	1.43
M24	13.46	0.65	14.11	16.66	1.31	17.97
M10	4.46	0.10	4.56	5.72	0.25	5.97
M28	2.54	—	2.54	0.31	—	0.31
M30	2.32	0.19	2.51	4.39	0.18	4.57
Unknowns ^b	0.27	0.26	0.53	—	0.18	0.18
PES	8.58	NA	8.58	3.65	NA	3.65
Total % of administered dose identified	89.38	4.13	93.51	84.45	6.17	90.62
<i>10 mg/kg bw</i>						
Active substance	18.05	—	18.05	20.35	—	20.35
M14	23.57	0.94	24.51	26.48	1.89	28.37
M22	2.51	—	2.51	2.58	—	2.58
M24	22.69	0.95	23.64	21.61	0.81	22.42
M10	3.46	—	3.46	5.00	0.08	5.08
M28	3.30	—	3.30	1.01	—	1.01
M30	3.54	0.12	3.66	5.89	0.17	5.06
Unknowns ^b	1.36	0.50	1.86	—	0.14	0.14
PES	14.16	NA	14.16	3.40	NA	3.40
Total % of administered dose identified	93.42	5.85	99.27	87.11	9.04	96.15
<i>Pulsed dose, 10 mg/kg bw</i>						
Active substance	18.25	—	18.25	17.16	—	17.16
M14	10.90	0.18	11.08	29.28	2.59	31.83
M22	7.17	0.12	7.29	3.06	—	3.06
M24	14.66	0.65	15.31	20.13	0.69	20.82
M10	3.57	—	3.57	3.38	0.13	3.51
M28	11.96	—	11.96	2.25	—	2.25
M30	5.35	—	5.35	4.29	—	4.29
Unknowns ^b	—	0.44	0.44	—	—	—
PES	10.60	NA	10.60	2.17	NA	2.17
Total % of administered dose identified	88.54	6.33	94.87	83.61	9.06	92.67

From Watts & Longacre (1998)

NA, Not applicable; PES, Post-extraction solids

The primary pathway probably involves demethylation of the A-ring methoxy moiety to form the corresponding phenol (M14), which is conjugated with glucuronic acid to form M16. Hydroxylation on the B-ring methyl moieties is also a significant metabolic pathway. Cleavage of methoxyfenozide to release either of the rings or the *t*-butyl group is only a minor pathway; none of the cleaved metabolites (M06, M07, M13, M32–36) were present at >2% of the dose. In males, however, cleaved metabolites represented up to about 50% of the metabolites found in urine. There was an indication that males cleaved more of the absorbed dose than did females, on the basis of urinary metabolite patterns.

Results for the animals receiving diets containing methoxyfenozide for 14 days plus a single dose of [¹⁴C]methoxyfenozide at 10 mg/kg bw by gavage showed evidence of induction of metabolism. Concentrations of M22, M28 and M30 increased, while concentrations of M14 and M24 were reduced relative to concentrations in animals that received only a single dose of [¹⁴C]methoxyfenozide at 10 mg/kg bw (Table 4) (Watts & Longacre, 1998).

1.3 Dermal absorption

The dermal absorption in vivo of methoxyfenozide formulated as an aqueous flowable liquid (RH-112,485 2F) or as a wettable powder (RH-112,485 280WP) was tested in rats in a study that was designed to comply with US EPA guidelines and GLP. The methoxyfenozide administered was uniformly labelled with ^{14}C on the methoxyphenyl ring; this is acceptable given the limited cleavage seen in studies of oral metabolism. To provide data on exposure to the concentrated product and in-use-dilutions, groups of four male Crl:CD BR rats received radiolabelled methoxyfenozide at three aqueous dilutions (0.025, 0.25, or 2.5% w/v), applied in a volume of 100 μl to a shaved area of about 10 cm^2 for 1, 10, or 24 h. Systemically absorbed methoxyfenozide was defined as the radiolabel found in the carcass, urine (plus urine funnel and cage washes), faeces, and whole blood.

For RH-112,485 2F, the total mean recovery of radiolabel in all groups ranged from 98% to 114%. After an exposure of 1, 10, or 24 h to ^{14}C -labelled RH-112,485 2F formulation diluted in water to a concentration of 2.5, 0.25, or 0.025% w/v, a small amount of radiolabel (<1–4%) was systemically absorbed.

For RH-112,485 280 WP, three animals with poor recoveries were excluded from further analysis. The total mean recovery of radiolabel in all groups ranged from 85% to 110%. After an exposure of 1, 10, or 24 h to ^{14}C -labelled RH-112,485 280 WP at a concentration of 2.5, 0.25, or 0.025% w/v, <1–2% of radiolabel was systemically absorbed.

Findings were similar for both formulations. The amount of radiolabel that was systemically absorbed did not increase linearly between the 10-h and 24-h exposure periods, indicating that most of the radiolabel that remained in or on the skin after washing was tightly bound and was not available for systemic absorption. This study shows that methoxyfenozide is poorly absorbed (<4%) after dermal exposure to either of the formulated products or in-use dilutions. The low rate of dermal absorption may be attributed to very low solubility in water (3.3 mg/l at 20°C) (Watts & Frederick, 1998).

2. Toxicological studies

2.1 Acute toxicity

(a) General toxicity

The acute toxicity of methoxyfenozide is summarized in Table 5. All studies used methoxyfenozide of 98% purity, and complied with the applicable OECD guideline and GLP. Methoxyfenozide is of low acute toxicity when administered by the oral, dermal and inhalation routes. No specific signs of toxicity were reported.

(b) Ocular and dermal irritation and dermal sensitization

Methoxyfenozide was not irritating to rabbit skin (Gingrich & Parno, 1995b) and produced only minimal, transient irritation to rabbit eyes (Gingrich & Parno, 1995c). Negative results were obtained in a Magnusson and Kligman maximization test for skin sensitization in guinea-pigs (Glaza, 1995).

Table 5. Acute toxicity of methoxyfenozide

Species	Strain	Sex	Route	Vehicle	LD ₅₀ (mg/kg bw) or LC ₅₀ (mg/l air)	Purity (%)	Reference
Rat	Crl:CD BR	Males & females	Oral	Methyl-cellulose	>5000	98	Gingrich & Parno (1995a)
Mouse	Crl:CD-1(ICR)BR	Males & females	Oral	Methyl-cellulose	>5000	98	
Rat	Crl:CD BR	Males & females	Dermal	Water	>5000	98	Procopio & Parno (1995)
Rat	Crl:CD BR	Males & females	Inhalation (4h, nose-only, MMAD, 6.3 µm)	None (dust aerosol)	>4.3 mg/l	98	Parno et al. (1998b) Bernacki & Fergusson (1995)

MMAD, mass median aerodynamic diameter

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In an initial study, groups of five female Crl:CD-1(ICR)BR mice were given diets containing methoxyfenozide (purity, 99.2%) at a concentration of 0, 50, 250, 1000, 2500 or 7000 mg/kg for 2 weeks. Limited investigations of clinical chemistry and haematology were performed in addition to routine investigations of mortality, clinical signs, body weight and food consumption. Microscopic examination was performed on the thyroid, liver, spleen, adrenals, kidney and pituitary from animals in the control group and the group receiving the highest dose. In addition, the liver from each mouse in all other treatment groups was also examined microscopically.

No clinical signs of ill health or reaction to treatment after 2 weeks of dietary exposure to methoxyfenozide were recorded. No treatment-related effects on haematology, clinical chemistry, body weight or food consumption were observed. The only findings of note were a statistically significant increase in relative liver weights at 7000 mg/kg and periportal hepatocyte hyperplasia at ≥ 2500 mg/kg. The no-observed-adverse-effect level (NOAEL) was 1000 mg/kg (equal to 217 mg/kg per day) on the basis of histopathological changes in the liver at 2500 mg/kg (Kaminski et al., 1993).

In a study that complied with GLP and the essential requirements of OECD guideline 408 (1981), groups of 10 male and 10 female Crl:CD-1(ICR)BR VAF Plus mice were given diets containing methoxyfenozide (purity, 99.2%) at a concentration of 0, 70, 700, 2500 and 7000 mg/kg for 3 months. These doses were equal to mean intakes of 0, 12, 113, 428 and 1149 mg/kg bw per day in males, and 0, 17, 165, 589, 1742 mg/kg bw per day in females. Active substance content, homogeneity and stability were satisfactorily demonstrated. All mice were observed daily for signs of ill health or reaction to treatment. Physical examinations and measurement of body weight and feed consumption were performed weekly. After 13 weeks, mice were bled for haematology (methaemoglobin formation was not investigated) and clinical chemistry analyses and were necropsied. An extensive histopathological examination was conducted for all animals in the control group and in the group receiving the highest dose. In addition, liver, lungs, kidneys and gross lesions from all animals from the other treatment groups were examined.

There were no deaths or substance-related clinical signs of toxicity. There were no significant substance-related effects on body weight or body-weight change in either sex at ≤ 2500 mg/kg. Cumulative body-weight gain after 4, 8 and 13 weeks was consistently reduced in both males (by 18–21%) and females (by 15–38%) at the highest dose. There were no notable substance-related haematological effects or changes in any clinical chemistry parameter. A statistically significant, small (4–6%) increase in mean cell volume and mean cell haemoglobin concentration for females at 7000 mg/kg was not considered to be an adverse effect, but might be an indication of a homeostatic response to effects on erythrocytes. There were no effects of toxicological concern on organ weights in either sex at any dose. A statistically significant increase in liver weight relative to body weight was not considered to be of toxicological concern because of its small magnitude (8%); this appeared to be secondary to reduced body weight (only a 3% increase in absolute liver weight) and there was no other evidence of hepatic toxicity in this study. Contrary to the previous 2-week study, no substance-related gross pathological changes or histopathological findings were observed in any tissues. The NOAEL for methoxyfenozide after 3 months of dietary feeding in mice was 2500 mg/kg (equal to 428 and 589 mg/kg bw per day for males and females, respectively) on the basis of reduced body-weight gain at 7000 mg/kg (equal to 1149 mg/kg bw per day) (Kaminski, Shuey & Gillette, 1995a).

Rats

In an initial study, groups of five male and five female CrI:CD®BR rats were given diets containing methoxyfenozide (purity, 90%) at concentrations of up to 20 000 mg/kg for 2 weeks (Table 6).

All rats were observed routinely for signs of ill health or reaction to treatment, body weight and feed consumption. After 2 weeks of treatment, blood samples were collected for measurement of haematology (including reticulocytes, but not methaemoglobin formation) and clinical chemistry parameters. Histological examination was limited to seven major organs at the highest dose and controls, except for the liver, thyroid and pituitary where there was evidence of substance-related effects at the highest dose.

There were no deaths and no effects on clinical signs, body weight or feed consumption were evident. No treatment-related adverse haematological effects were observed in either sex at any dose. No clear treatment-related clinical chemistry changes were seen in males at doses of up to 5000 mg/kg or in females at doses of up to 20 000 mg/kg. A treatment-related decrease (by 47%, statistically significant) in triglyceride concentrations was

Table 6. Methoxyfenozide content of diets fed to rats for 13 weeks

Dietary concentration (mg/kg)	Mixing procedure	Mean intake (mg/kg bw per day)	
		Males	Females
0		0	0
50	Acetone/powder mixing procedure	5	5
250	Acetone/powder mixing procedure	24	25
1000	Acetone/powder mixing procedure	101	99
1000	Powder/powder mixing procedure	99	98
5000	Acetone/powder mixing procedure	507	513
5000	Powder/powder mixing procedure	502	489
20000	Acetone/powder mixing procedure	1977	1999

From Kyle et al. (1992)

observed in males at 20 000 mg/kg. Increases in absolute and relative weights of liver (14–23%) and adrenals (up to 30%) were noted at >5000 mg/kg. Minimal to slight hepatocyte hypertrophy was observed in all rats at 20 000 mg/kg and in three females at 5000 mg/kg. There was evidence of single cell necrosis in the livers of animals receiving 20 000 mg/kg. A dose-related minimal to slight hypertrophy and/or hyperplasia of the follicular cells of the thyroid occurred in male and female rats at ≥ 1000 mg/kg. Minimal hypertrophy of the zona fasciculata of the adrenal cortex was seen in three females at 20 000 mg/kg. Isolated findings of adrenal hypertrophy in females at 1000 and 5000 mg/kg were of questionable significance. The NOAEL was 250 mg/kg (equal to 24 mg/kg per day) on the basis of follicular cell hypertrophy and/or hyperplasia of the thyroid in both sexes at 1000 mg/kg (equal to 98 mg/kg bw per day) (Kyle et al., 1992).

In a study complying with GLP and the essential requirements of OECD guideline 408 (1981), groups of 10 male and 10 female Crl:CD BR rats were given diets containing methoxyfenozide (purity, 99.2%) at a concentration of 0, 50, 250, 1000, 5000 or 20 000 mg/kg for 90 days. These doses were equal to mean intakes of 0, 3.4, 17, 69, 353 and 1369 mg/kg bw per day for males, and 0, 3.7, 19, 72, 379 and 1531 mg/kg bw per day for females. Adequate stability, homogeneity and achieved concentration were demonstrated. Body weights and food consumption were measured weekly. Blood samples from non-fasted were taken at termination for the assessment of haematological (excluding methaemoglobin formation) and clinical chemistry parameters. Urine analysis was performed before termination. Ophthalmoscopy was performed before dosing and before termination. A comprehensive list of tissues from animals in the control group and in the group receiving the highest dose was examined microscopically; liver, lungs, kidneys and gross lesions were investigated in animals at the intermediate dose.

One female at 1000 mg/kg was found dead during week 7. No clinical signs of toxicity were noted in any animal. No notable effects were seen on body weight, body-weight gain, food consumption or ophthalmoscopy. Haematological findings indicative of mild anaemia were noted at the highest dose (Table 7). Plasma aspartate transaminase, alanine transaminase and alkaline phosphatase activities were decreased in treated groups; however, these findings were not considered to be of toxicological significance. Slight changes in other clinical chemistry values suggested mild hepatotoxicity at 20 000 mg/kg (Table 7). There was an indication of a slight increase in urinary protein concentrations in females at 5000 and 20 000 mg/kg, but in the absence of any related pathological findings this was not considered to be an adverse finding. No gross treatment-related findings were seen at necropsy. Liver weights were increased, and there was evidence of periportal hepatocyte hypertrophy in both sexes at doses of ≥ 5000 mg/kg (Table 7). Thyroid weights were also increased slightly in males at the highest dose, but in the absence of histopathological correlates this is of unclear toxicological significance. The NOAEL was 1000 mg/kg (equal to 69 mg/kg bw per day) on the basis of increased relative liver weights (>10%) and associated hypertrophy at ≥ 5000 mg/kg (Anderson et al., 1995).

Dogs

Five reports of dietary studies in dogs were submitted: two 2-week studies, a 90-day study, a 1-year study and a study of reversibility of blood effects. All studies, except the first 2-week study, were conducted by the same laboratory.

Table 7. Findings in rats given diets containing methoxyfenozide for 90 days

Parameter	Sex	Dietary concentration (mg/kg)					
		0	50	250	1000	5000	20 000
Mean intake (mg/kg bw per day)	M	—	3.4	17	69	353	1 369
	F	—	3.7	19	72	379	1 531
Terminal body weight (g)	M	587.8	585.1	557.6	587.0	573.1	574.2
	F	330.0	323.8	356.0	323.4	334.1	324.1
Body-weight gain (g)	M	479.6	480.0	449.7	479.5	468.9	463.9
		214.3	214.2	242.3	206.3	222.8	207.9
<i>Haematology</i>							
Erythrocytes (10 ⁶ /μl)	M	8.22	8.37	8.11	8.49	8.06	7.95
	F	7.48	7.36	7.09	7.46	7.27	6.92*
Erythrocyte volume fraction (l/l)	M	0.470	0.472	0.460	0.479	0.469	0.458
	F	0.450	0.446	0.433	0.456	0.441	0.426
Haemoglobin (g/dl)	M	158	15.9	15.7	16.0	15.9	15.5
	F	15.7	15.6	15.3	15.8	15.3	14.6*
<i>Clinical chemistry</i>							
AST (U/l)	M	124	130	110	108	105	115
	F	123	110	97	101	94	82*
ALT (U/l)	M	53	62	46	51	52	57
	F	66	53	54	52	50	47*
AP (U/l)	M	360	392	356	274	313	247*
	F	358	312	296	248	245*	204*
Triglycerides (mg/dl)	M	131	149	119	137	147	168
	F	81	70	100	69	90	85
Cholesterol (mg/dl)	M	67	70	63	62	67	64
	F	76	71	76	77	69	63*
Albumin (g/dl)	M	3.8	4.0	3.8	3.9	4.0	4.2*
	F	4.4	4.2	4.3	4.2	4.2	4.1
Total protein (g/dl)	M	5.9	6.2	6.1	6.2	6.2	6.4*
	F	6.4	6.2	6.4	6.3	6.3	6.4
Albumin : globulin ratio	M	1.8	1.8	1.8	1.8	1.8	1.8
	F	2.2	2.1	2.1	2.0	1.9	1.8*
<i>Organ weights</i>							
Liver (g)	M	20.5	21.6	20.2	21.4	22.0	23.1
	F	11.8	11.3	12.5	11.2	12.9	13.0
Liver (% bw)	M	3.39	3.59	3.53	3.57	3.77*	3.94*
						(11%)	
Thyroid (g)	F	3.53	3.41	3.41	3.39	3.78	4.06*
	M	0.031	0.031	0.030	0.030	0.034	0.037
Thyroid (% bw) × 100	F	0.023	0.022	0.023	0.021	0.024	0.022
	M	0.526	0.526	0.524	0.493	0.582	0.623
	F	0.684	0.664	0.642	0.649	0.705	0.657
<i>Liver histopathology</i>							
Hypertrophy (a = slight, b = moderate)	M	—	—	—	—	6a; 4b	10b
	F	—	—	—	—	10a	10b

From Anderson et al. (1995)
M, male; F, female; AST, aspartate transaminase; ALT, alanine transaminase; AP, alkaline phosphatase
**p* < 0.05

In a 2-week dietary study conducted in 1992, a final report was issued in 1994 and a revised report was issued in 1995; the latter included amendments to the interpretation of the haematology, clinical chemistry and organ weight data. The present summary refers to the revised study report, which complied with GLP. Groups of two male and two female beagle dogs (aged 6–8 months at the start of dosing) were given diets containing methoxyfenozide (purity, 99.2%) at concentrations of up to 30 000 mg/kg (Table 8), as 400 g of food per day. Body weight was measured before dosing and on days 1, 2, 4, 7 and 14. Food consumption was measured daily. Blood samples for haematology (including methaemoglobin formation and reticulocytes) and clinical chemistry examination were

Table 8. Dietary concentrations of methoxyfenozide administered in a 2-week study in dogs

Group	Dietary concentration (mg/kg)	Mean intake (mg/kg bw per day)	
		Male	Female
1	0	0	0
2	300	13	16
3	3 500	154	157
4	7 000	308	299
5	15 000	603	608
6	30 000	1225	1130

From Nuttall & Kelly (1995)

taken after about 18 h without food before dosing and in week 2 (this might permit some recovery of levels of methaemoglobin formation). At necropsy major organs were weighed, but no histopathological investigations were performed.

There were no deaths, no treatment-related clinical signs and no notable changes in food consumption or body-weight gain.

Treatment-related changes in erythrocyte cell parameters, indicative of haemolytic anaemia, were recorded for animals of each sex receiving methoxyfenozide at ≥ 3500 mg/kg. However, not all changes in haemolytic parameters were dose-dependent. Particularly notable was a clear dose-related increase in the mean percentage of erythrocytes with Heinz bodies: males, 0% of controls, 13% at 3500 mg/kg, rising to 41% at 30 000 mg/kg; females, 0% of controls, 17% at 3500 mg/kg, rising to 33% at 30 000 mg/kg. The mean percentage of methaemoglobin was increased, but there was no clear dose-response: males, 0.8% for controls, 1.5–3.4% at ≥ 3500 mg/kg; females, 1.0% for controls, 1.7–3.5% at ≥ 3500 mg/kg. A slight to moderate incidence of Howell-Jolly bodies was seen in all males at ≥ 3500 mg/kg and in all females at ≥ 7000 mg/kg. The only treatment-related effect was an increase in plasma total bilirubin concentrations in most dogs receiving ≥ 3500 mg/kg when compared with values before dosing and for controls. The spleen was the only organ investigated for which there were indications of a treatment-related effect on weight. Increases in relative and absolute weights were seen in both sexes at ≥ 3500 mg/kg and in males at 300 mg/kg (Table 9).

The NOAEL was 300 mg/kg (equal to 13 mg/kg bw per day) on the basis of evidence of haemolytic anaemia (haematological changes, increased serum bilirubin, increased spleen weight), in both sexes at 3500 mg/kg. The increased spleen weight at 300 mg/kg in males is not considered to be adverse in the absence of effects on erythrocytes (Nuttall & Kelly, 1995).

Groups of two male and two female beagle dogs were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0, 500, 5000, 15 000 or 30 000 mg/kg for 2 weeks. Mean intakes were 0, 18, 202, 509 and 1003 mg/kg bw per day for males, and 20, 196, 757 and 1186 mg/kg bw per day for females. The study complied with GLP. All dogs were observed routinely for signs of ill health or reaction to treatment, and body weight and food consumption were recorded. All dogs were offered 400 g of feed daily, beginning with a 2-week period before dosing. During the first week of this 2-week period and before

Table 9. Spleen weights in dogs given diets containing methoxyfenozide for 2 weeks

Dietary concentration (mg/kg)	Body weight (g)	Spleen weight (g)	Spleen: body weight (%)	Spleen: body weight (% change versus control)
<i>Males</i>				
0	9850	25.3	0.26	
300	8600	30.3	0.35	+35%
3500	8680	58.4	0.67	+111%
7000	8700	42.9	0.51	+98%
15000	8600	77.0	0.89	+249%
30000	10000	63.2	0.62	+142%
<i>Females</i>				
0	7550	25.1	0.33	
300	7500	23.6	0.31	-6%
3500	7725	40.3	0.52	+57%
7000	7650	63.9	0.85	+154%
15000	7675	45.9	0.63	+88%
30000	7350	38.8	0.53	+58%

From Nuttall & Kelly (1995)

Table 10. Reticulocyte counts in dogs given diets containing methoxyfenozide for 2 weeks

Dietary concentration (mg/kg)	Reticulocytes (%)		Mean % change compared with:	
	Day -12	Day 16	Before dosing	Concurrent control
<i>Males</i>				
0	0.0; 0.2	0.7; 0.2	+400	—
500	0.4; 0.0	0.8; 0.6	+250	+40
5000	0.6; 0.8	1.5; 1.0	+86	+160
15000	0.2; 0.6	2.0; 1.6	+350	+260
30000	0.0; 0.2	1.0; 2.6	+1700	+260
<i>Females</i>				
0	0.4; 0.2	1.2; 0.6	+200	—
500	0.0; 0.4	0.4; 1.2	+300	-11
5000	0.2; 0.4	2.8; 2.0	+700	+167
15000	0.2; 0.4	4.6; 9.1	+2200	+667
30000	0.0; 0.2	6.6; 3.6	+5000	+467

From Vandenberghe & Gillette (1995)

necropsy, blood samples were collected from all dogs for haematology (including methaemoglobin formation and reticulocytes) and clinical chemistry analyses) were saved in 10% neutral buffered formalin. Liver and spleen were weighed. Histopathological evaluation was performed on liver, spleen, bone marrow and all gross lesions from all dogs.

There were no compound-related deaths, clinical signs of systemic toxicity, and no effects on body weight or feed consumption. In females at 15 000 and 30 000 mg/kg, changes seen included a decrease in erythrocyte counts (about 30%), haemoglobin concentration (about 25%) and erythrocyte volume fraction (about 25%), an increase in methaemoglobin formation (about 300%) and a significant increase in the percentage of reticulocytes (by 500–5000%). Other effects observed included Heinz bodies and nucleated erythrocytes. Minimal changes in erythrocyte counts, haemoglobin concentration, erythrocyte volume fraction and reticulocyte counts were observed in both sexes at 5000 mg/kg and in males at 15 000 and 30 000 mg/kg. Given the small group size, these latter changes are difficult to interpret, but overall there would seem to be a substance-related increase in the percentage of reticulocytes at ≥5000 mg/kg in females (Table 10). There was some evidence for a slight

increase in total bilirubin in females at the highest dose, but the variability in values before dosing makes this an equivocal finding.

Relative spleen weight was increased compared with that of controls at most doses in both sexes; the lack of a clear dose-related response in either sex makes it questionable whether the response was treatment related (Table 11). Histopathological findings were limited to minimal haemosiderin accumulation in Kupffer cells in the livers of females at 15 000 and 30 000 mg/kg. There were no histopathological findings in the spleen.

The NOAEL was 500 mg/kg (equal to 20 mg/kg bw) on the basis of increased reticulocytes in females at 5000 mg/kg (196 mg/kg bw per day) (Vandenberghe & Gillette, 1995).

Groups of four male and four female beagle dogs (aged 5–6 months at the start of dosing) were given diets containing methoxyfenozide (purity, 99.8%) at a concentration of 0, 15, 50, 500 or 5000 mg/kg for 13 weeks. Owing to an apparent lack of substance-related effects at all doses, dogs from the group receiving 15 mg/kg were dosed for a further 2 weeks at 15 mg/kg and then for 6 weeks at 15 000 mg/kg. The mean dietary intake during dosing at the higher concentration was 422 mg/kg bw per day in males and 460 mg/kg bw per day in females. The lack of concurrent negative controls for these animals at the highest dose limits interpretation of the results, but for some parameters a comparison can be made of findings before and after the increase in dose. The study complied with GLP and with OECD guideline 409 (1998); the absence of toxicity at the highest dose was acceptable given the findings in the 2-week studies.

Stability, homogeneity and achieved dietary content were satisfactory. Mean intakes of methoxyfenozide are shown in Table 12. Dogs had access to 400 g of food per day for 2 h. Dogs were observed routinely for mortality, clinical signs, body weight and food consumption. Fasted blood samples were taken for the assessment of haematological (including methaemoglobin formation, erythrocyte morphology and reticulocyte count) and clinical chemistry parameters before the start of dosing and during weeks 7 and 13, with samples being taken from the extended dosing group after dosing at 15 000 mg/kg for 16 and 38 days. Ophthalmoscopy was performed before the start of dosing and before termination. Urine analysis was performed at termination. Weights of the adrenals, liver, spleen,

Table 11. Spleen weight in dogs given diets containing methoxyfenozide for 2 weeks

Dietary concentration (mg/kg)	Mean body weight (g)	Spleen weight (g)	Spleen:body weight (%)	Spleen:body weight (% change versus control)
<i>Males</i>				
0	8970	28.500	0.318	—
500	9200	47.200	0.530	+67
5000	8680	58.350	0.671	+111
15000	8800	40.950	0.469	+47
30000	8730	36.600	0.428	+35
<i>Females</i>				
0	7910	24.600	0.311	—
500	7630	37.050	0.484	+56
5000	7190	33.500	0.461	+48
15000	7870	23.650	0.299	—4
30000	7680	31.900	0.415	+33

From Vandenberghe & Gillette (1995)

Table 12. Findings in dogs given diets containing methoxyfenozide for 90 days

Parameter	Dietary concentration (mg/kg)				
	0	15	50	500	5000
<i>Males</i>					
Intakes (mg/kg bw per day)	0	0.6	2.0	21	198
Absolute kidney weight (g)	59	—	54	53	50
Relative liver weight (% body weight)	2.8	—	3.0	3.2	3.2
Body-weight gain (g)					
Weeks 0–6	2000	1675	1500	1400	1775
Weeks 6–13	1650	1600	1150	1800	1500
Platelets ($10^3/\mu\text{l}$)					
Before start of dosing	393	356	390	357	401
Day 45	317	342	393	332	393
Day 87	301	302	348	323	366
Erythrocyte count ($10^6/\mu\text{l}$)					
Before start of dosing	6.14	5.95	6.32	5.78	5.84
Day 45	5.84	5.72	5.94	5.38	5.38
Day 87	7.29	6.12*	6.07*	5.88*	6.07*
Haemoglobin (g/dl)					
Before start of dosing	12.7	13.4	13.8	12.7	12.6
Day 45	12.1	12.8	13.3	12.1	12.0
Day 87	15.0	13.7	13.6	13.0*	13.1*
Erythrocyte volume fraction (l/l)					
Before start of dosing	0.364	0.379	0.394	0.362	0.363
Day 45	0.350	0.366	0.376	0.343	0.341
Day 87	0.440	0.394	0.387	0.371*	0.384
Methaemoglobin (%)					
Before start of dosing	1.0	0.7	0.8	1.0	0.8
Day 45	1.0	0.9	0.9	1.0	1.0
Day 87	0.7	0.8	0.8	0.8	1.0*
<i>Females</i>					
Intakes (mg/kg bw per day)	0	0.6	1.9	20	209
Absolute kidney weight (g)	44	—	44	53*	42
Relative liver weight (% body weight)	2.7	—	2.8	3.1	3.1
Body-weight gain (g)					
Weeks 0–6	1825	1650	1800	1850	1300*
Weeks 6–13	1150	1175	1025	1400	1125
Platelet ($10^3/\mu\text{l}$)					
Before start of dosing	444	375	372	402	400
Day 45	410	335	361	352	364
Day 87	351	332	310	325	300
Erythrocyte count ($10^6/\mu\text{l}$)					
Before start of dosing	6.38	6.02	6.02	5.81	6.13
Day 45	6.19	5.83	6.24	5.48	5.93
Day 87	6.90	6.58	6.77	6.18	6.35
Haemoglobin (g/dl)					
Before start of dosing	13.3	13.5	13.4	13.2	13.5
Day 45	13.3	13.3	13.9	12.5	13.5
Day 87	14.7	14.6	14.9	13.6	14.2
Erythrocyte volume fraction (l/l)					
Before start of dosing	0.386	0.387	0.382	0.374	0.390
Day 45	0.381	0.381	0.399	0.356	0.383
Day 87	0.426	0.424	0.429	0.394	0.403
Methaemoglobin (%)					
Before start of dosing	0.9	0.9	0.9	1.0	0.9
Day 45	0.9	0.9	0.9	1.1	0.9
Day 87	0.7	0.8	0.8	0.9	0.7

From Kaminski, Shuey & Lomax (1995b)

* Values are means, $n = 4$ * Significant difference from control, $p < 0.05$; analysis of covariance followed by Dunnett t -test

kidneys, testes, brain and thyroids were recorded at necropsy. A comprehensive list of tissues from all animals was examined histopathologically.

All dogs survived to termination and no substance-related clinical signs were observed. Mean body weight and body-weight gains of females at 5000 mg/kg were lower than controls at the start of the study and attained statistical significance at a number of time-points, but body-weight gain between weeks 6 and 13 was similar to that of controls (Table 12). These findings were primarily, but not entirely, attributable to one dog that gained only 2.0 kg (control range, 2.4–3.1 kg). No effects on body weight were observed in males. Interpretation of the body weight data for dogs dosed at 15 000 mg/kg is difficult in the absence of concurrent controls. No effects were seen on food consumption by either sex during the main 90-day study. There was also no change in mean daily food consumption of animals for which the dose administered was increased from 15 mg/kg to 15 000 mg/kg.

No consistent effects were seen on haematological parameters during the main 90-day study. Although there were some statistically significant changes, none were considered to be substance-related because of the lack of change from values before the start of dosing and/or lack of convincing time- or dose-response relationship. The statistically significant reduction in erythrocyte count at all doses in males on day 87 was associated with a high value for controls and showed no dose-response relationship (Table 12). The impact of fasting on methaemoglobin concentrations is uncertain. There were no apparent haematological effects in animals for which the dose was increased from 15 mg/kg to 15 000 mg/kg. There were no notable changes in clinical chemistry parameters when variations in values before the start of dosing were taken into consideration. Urine analysis results indicated a slight increase in specific gravity and protein content in both sexes at 5000 mg/kg. Ophthalmoscopy findings were similar in all groups.

Relative liver weights were higher at 500 and 5000 mg/kg in both sexes compared with those of the controls, but there was no clear dose-response relationship (Table 12). Absolute liver weights were not increased in the satellite group receiving 15 000 mg/kg. Absolute and relative kidney weights were decreased slightly in males at 50, 500 and 5000 mg/kg (Table 12). Individual kidney weights (absolute and relative) at 5000 mg/kg were mostly below the range of values for the controls, which when taken with the urine analysis results, indicates a possible marginal effect at 5000 mg/kg. After exposure to 15 000 mg/kg, relative kidney weights for males were mostly below the range of values for controls from the main part of the study. An apparent decrease in absolute and relative spleen weight in males at 50–5000 mg/kg is attributed to one control male with a large spleen. No substance-related gross or microscopic findings were noted at necropsy of animals at the end of the main 90-day study or at the end of exposure to 15 000 mg/kg. The absence of any animals with pigment in the liver or spleen is notable.

The NOAEL was 5000 mg/kg (198–209 mg/kg bw per day). The slight effects on body weights, haematology, urine analysis and organ weights are not considered to be clearly adverse, given the absence of histopathological findings and the variability in values before the start of dosing (Kaminski, Shuey & Lomax, 1995b).

In a study that essentially complied with OECD guideline 452 (1981) and was performed according to GLP, groups of four male and four female beagle dogs (aged 5–6 months at the start of dosing) were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0, 60, 300, 3000, or 30 000 mg/kg (equal to 0, 2.2, 9.8, 106 or 1152

mg/kg bw per day in males, and 0, 2.2, 12.6, 111 or 1199 mg/kg bw per day in females) for 52 weeks. Dogs had access to about 400 g of food for 2 h per day (except for one occasion when food had to be left in the cages overnight). Adequate homogeneity, stability and content of the diets were demonstrated. Dogs were observed routinely for mortality, signs of ill health, body weight and food consumption. Urine analysis was conducted for all dogs before the start of testing, and after 3, 6, and 12 months of treatment. Blood samples were collected from all (fasted) dogs for haematology (including methaemoglobin formation, but not morphology or reticulocyte count) and clinical chemistry analyses before the start of testing, and after 3, 6, and 12 months of treatment. Ophthalmology examinations were performed on all dogs before the start of testing and after 12 months of treatment. At termination, all dogs were killed and necropsied, major organs were weighed and an extensive range of tissues from all animals was examined histologically.

There were no deaths or clinical signs of toxicity. Body-weight gain was reduced at 30 000 mg/kg in males at the start of the study, but was similar to that of controls for the final 9 months of the study (Table 13). In females, body-weight gain was reduced at 30 000 mg/kg over the latter half of the study, primarily as a result of weight loss by one animal (Table 13). Food consumption was similar in all groups.

The percentage of methaemoglobin was statistically significantly increased at 30 000 mg/kg in both sexes compared with controls (Table 14). There was also evidence for a slight increase in methaemoglobin concentration at 3000 mg/kg in both sexes when compared with mean and individual values before the start of dosing. Although the maximum mean percentage of methaemoglobin (2.8%) at 30 000 mg/kg was not clearly adverse relative to the background level in humans (2–12%) (Bell et al., 1972), the use of fasted samples could mean that the peak percentage in dogs was >2.8%. Decreases were seen in erythrocyte parameters at 3000 and 30 000 mg/kg in both sexes (Table 14). Although many of the changes at 3000 mg/kg were not statistically significant when compared with controls, the changes were evident relative to values for the group before the start of testing (Table 14). There was also a very minimal increase in nucleated erythrocytes in both sexes at 30 000 mg/kg. The pattern of changes in haematology is consistent with methaemoglobin-induced anaemia. Platelet numbers were increased in both sexes at 3000 mg/kg (by about 50%) and 30 000 mg/kg (by about 85%) compared with numbers for controls. There were no effects on white blood cells. Total bilirubin in blood and urine was increased at 3000 and

Table 13. Mean body-weight gain (range) in dogs given diets containing methoxyfenozide for 1 year

	Dietary concentration (mg/kg)				
	0	60	300	3000	30 000
<i>Males</i>					
Weeks 0–13	3042 (2755–3307)	2520 (2157–2826)	2928 (1912–3877)	2888 (2470–3383)	2518 (2019–3174)
Weeks 0–52	4022 (3530–4218)	3571 (3221–3960)	3971 (2425–5958)	3752 (3511–3967)	3111 (2298–3627)
<i>Females</i>					
Weeks 0–26	2474 (1981–2982)	2684 (2431–2885)	2227 (1303–3427)	2834 (1890–3483)	2578 (1766–3157)
Weeks 0–52	2782 (2362–3077)	2641 (2333–3272)	2446 (1463–3621)	2635 (1985–3311)	2348 (1391–2791)

From Morrison & Shuey (1997)

Table 14. Haematology results in dogs given diets containing methoxyfenozide for 1 year

Parameter	Dietary concentration (mg/kg)				
	0	60	300	3000	30 000
<i>Males</i>					
Platelet ($10^3/\mu\text{l}$)					
Before start of dosing	378	319	349	425	405
Day 93	345	298	328	542*	650*
Day 184	305	267	294	497*	581*
Day 366	309	255	309	468*	605*
Erythrocyte count ($10^6/\mu\text{l}$)					
Before start of dosing	6.20	5.93	5.67	5.64	6.46
Day 93	6.53	6.25	6.61	5.60*	5.57*
Day 184	6.55	6.17	6.38	5.77	5.83
Day 366	6.81	6.99	6.92	6.24	6.52
Haemoglobin (g/dl)					
Before start of dosing	13.2	12.8	12.4	12.8	13.8
Day 93	13.9	13.6	14.5	12.9	12.4
Day 184	14.0	13.3	13.9	13.1	13.2
Day 366	14.5	14.9	15.2	14.3	14.4
Erythrocyte volume fraction (l/l)					
Before start of dosing	0.388	0.371	0.362	0.368	0.399
Day 93	0.407	0.398	0.425	0.377	0.362
Day 184	0.408	0.394	0.417	0.390	0.383
Day 366	0.425	0.442	0.442	0.416	0.422
Methaemoglobin (%)					
Before start of dosing	0.6	0.7	0.8	0.6	0.7
Day 93	0.6	0.7	0.8	1.0	2.8*
Day 184	0.6	0.7	0.6	1.0	2.2*
Day 366	0.7	0.9	0.9	1.1	1.9*
<i>Females</i>					
Platelet ($10^3/\mu\text{l}$)					
Before start of dosing	418	326	385	373	333
Day 93	343	286	420	430	562*
Day 184	332	298	388	430	560*
Day 366	368	372	421	434	667*
Erythrocyte count ($10^6/\mu\text{l}$)					
Before start of dosing	6.62	5.88	6.30	5.64	6.06
Day 93	6.76	6.35	6.36	5.54*	5.07*
Day 184	6.96	6.20	6.17	5.54*	5.23*
Day 366	7.49	6.42	6.75	6.09*	5.84*
Haemoglobin (g/dl)					
Before start of dosing	14.0	12.9	13.6	12.2	13.2
Day 93	14.4	14.2	13.8	12.3	12.0
Day 184	14.9	13.9	13.6	12.7*	12.3*
Day 366	16.0	14.6	15.0	13.7	13.8
Erythrocyte volume fraction (l/l)					
Before start of dosing	0.412	0.383	0.397	0.357	0.386
Day 93	0.424	0.413	0.405	0.359	0.344*
Day 184	0.437	0.410	0.400	0.366*	0.363*
Day 366	0.469	0.424	0.434	0.395	0.399
Methaemoglobin (%)					
Before start of dosing	0.8	0.7	0.8	0.8	0.9
Day 93	0.6	0.8	0.9	1.1	2.5*
Day 184	0.8	0.7	0.8	1.0	2.8*
Day 366	0.8	0.8	1.4	1.3	1.9*

From Morrison & Shuey (1997)

* $p < 0.05$

30 000 mg/kg in both sexes. There was no effect on blood urea nitrogen, urine specific gravity or protein content, or on ophthalmological findings.

There were increases in mean absolute and relative liver weights (by 13 and 24%, respectively) and in mean absolute and relative thyroid weights (by 43 and 63%, respectively) of males at 30 000 mg/kg. Kidney weight was unaffected by treatment. There were no treatment-related gross pathological findings. There were no histopathological correlates to the increased liver and thyroid weights, although increased iron positive pigment was seen in splenic and hepatic macrophages in animals receiving 30 000 mg/kg. In the bone marrow, increased cellularity characterized by decreased fat vacuoles and increased haematopoietic cells (primarily erythrocytes and erythrocyte precursors) was observed at 30 000 mg/kg. The histopathological changes were consistent with the normal response to haemolytic anaemia. There were no substance-related histopathological effects on the thyroid or kidney.

The NOAEL was 300 mg/kg (equal to 9.8 mg/kg bw per day) on the basis of changes in liver hypertrophy, erythrocyte parameters and increased blood platelet count at 3000 mg/kg (equal to 106 mg/kg bw per day) (Morrison & Shuey, 1997).

A study complying with GLP was designed to investigate the reversibility of blood effects seen in the 1-year study in dogs. Groups of four male beagle dogs (aged 10–11 months at the start of dosing) were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0 or 30 000 mg/kg for 4 weeks (the dosing phase). The dogs were selected from the control group and group receiving the lowest dose in a previously conducted 13-week study of dermal application with another test material. The dogs had access to about 400 g of food for 2 h per day. Acceptable stability, homogeneity and achieved content of the diet were demonstrated. The achieved intakes were 0 and 1036 mg/kg bw per day. After 4 weeks of treatment, all animals were maintained on untreated diet for 4 additional weeks (the recovery phase).

All dogs were observed routinely for mortality, clinical signs, feed consumption and body weights. Blood samples (fasting not specified) were collected from all dogs for haematology analyses (reticulocytes not counted) before the start of treatment, after 4 weeks of treatment, and after 2 and 4 weeks of recovery. At the end of the recovery phase, animals were returned to the animal care unit. No mortalities or clinical signs indicative of systemic toxicity were seen. Body-weight gain and food consumption were unaffected by treatment. Methoxyfenozide produced a range of haematological effects after 4 weeks of dietary administration at 30 000 mg/kg bw (Table 15). Clear evidence of reversibility was demonstrated after the 4-week recovery phase (Table 15) (Bannister & Morrison, 1998).

(b) *Dermal exposure*

Rats

This study of toxicity exceeded the basic requirements of OECD guideline 410 (1981), notably in terms of the number of animals used and the extent of the histopathological examination. The study complied with GLP. Methoxyfenozide (purity, 98%), a powder, was moistened with tap water (1:1, w/v) and applied to the shaved intact skin (about 10% of the total body surface area) of Crl:CD®BR rats (10 of each sex per group) at a dose of 0, 75, 300 or 1000 mg/kg bw per day for 5 days per week over a 4-week period (20 exposures). The exposure site was covered with an occlusive dressing, which was removed after 6 h and

Table 15. Haematology findings in male dogs given diets containing methoxyfenozide (recovery study)

Parameter	Dietary concentration (mg/kg)	
	0	30 000
Methaemoglobin (%)		
Before start of dosing phase—day 0	0.7	0.8
Dosing phase—day 28	0.8	2.2*
Recovery phase—day 14	0.9	1.4
Recovery phase—day 28	0.9	1.0
Mean cell volume (μm^3)		
Before start of dosing phase—day 0	67.8	68.6
Dosing phase—day 28	66.8	72.6*
Recovery phase—day 14	66.7	71.6*
Recovery phase—day 28	67.0	70.3
Mean cell haemoglobin (pg)		
Before start of dosing phase—day 0	23.5	23.9
Dosing phase—day 28	23.3	25.2*
Recovery phase—day 14	23.2	24.8*
Recovery phase—day 28	22.9	24.3
Platelets ($10^3/\mu\text{l}$)		
Before start of dosing phase—day 0	339	333
Dosing phase—day 28	338	487
Recovery phase—day 14	334	416
Recovery phase—day 28	326	362
Haemoglobin (g/dl)		
Before start of dosing phase—day 0	15.6	16.3
Dosing phase—day 28	15.8	14.9*
Recovery phase—day 14	16.0	16.2
Recovery phase—day 28	16.5	17.0
Erythrocyte count ($10^6/\mu\text{l}$)		
Before start of dosing phase—day 0	6.63	6.86
Dosing phase—day 28	6.79	5.91*
Recovery phase—day 14	6.91	6.54
Recovery phase—day 28	7.18	7.01
Erythrocyte volume fraction (l/l)		
Before start of dosing phase—day 0	0.449	0.470
Dosing phase—day 28	0.453	0.428*
Recovery phase—day 14	0.461	0.468
Recovery phase—day 28	0.480	0.492

From Bannister & Morrison (1998)

* $p < 0.05$; Dunnett t -test

the exposure site was washed with soap solution and then wiped. Animals were observed routinely for mortality, signs of ill health, reaction to treatment, body weight and food consumption. Application sites were evaluated for skin irritation before each daily treatment and at terminal sacrifice. Haematology (including methaemoglobin measurement, but not reticulocyte count) and clinical chemistry were performed at terminal sacrifice. An indirect ophthalmoscopic examination was performed before initiation of dosing and at the end of the dosing period. An extensive histopathological examination (including treated and non-treated skin) was conducted for all control animals and animals at the highest dose. In addition, kidneys, liver, lungs and all gross lesions from all animals were examined histopathologically.

All rats survived to termination. There was no evidence of substance-related effects on clinical signs, skin irritation, ocular effects, haematology, clinical chemistry, organ

weight or histopathology. A reduction in food consumption (13%) in week 4 and reduced body-weight gain (16% between days 0–28) in males at 1000 mg/kg bw per day was not considered to be an effect of clear toxicological importance; there was no statistically significant decrease in body weight and no effect on food consumption or body-weight gain was seen in females. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Parno et al., 1998a).

2.3 *Long-term studies of toxicity and carcinogenicity*

Mice

Groups of 60 male and 60 female Crl:CD-1®(ICR)BR (VAF Plus) mice were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0, 70, 2800, or 7000 mg/kg for 18 months. These concentrations resulted in mean intakes of 0, 10.0, 405 or 1020 mg/kg bw per day in males, and 0, 12.8, 529 or 1354 mg/kg bw per day in females. Mice had access to food ad libitum and were housed individually. Satisfactory stability, homogeneity and content of the test material were demonstrated. Mice were observed routinely for mortality, ill health, reaction to treatment, body weight and feed consumption. After 12 and 18 months of treatment, white blood cell differential counts were conducted for all control mice and mice at the highest dose. An extensive histopathological examination was performed for these mice, as well as on mice that died in groups receiving the other doses. Microscopic examination for survivors at the lowest and intermediate doses was limited to liver, kidneys, lungs and gross lesions. The study report included an independent peer review that confirmed the conclusions of the original pathologist. The study complied with OECD guideline 451 (1981) and GLP.

No effects on survival were observed in mice at any dose. A total of 43–48 males per group and 47–52 females per group survived to termination. There were no compound-related clinical signs indicative of systemic toxicity in any of the treatment groups. There were no substance-related effects on body weight, cumulative body-weight gain, food consumption, white blood cell counts or non-neoplastic pathology findings. A slight (8–10%) but statistically significant reduction in kidney weight in males at 2800 and 7000 mg/kg was not considered to be substance-related, in the absence of a dose–response relationship, the lack of an effect on relative kidney weight and no associated histopathological findings. A slight dose-related increase in absolute liver weight (9–13%) and relative liver weight (12–18%) in males at 2800 and 7000 mg/kg was considered to be of no toxicological importance because the increases were not statistically significant, were not seen in females and there were no related histopathological findings. A slight increase in the incidence of extramedullary haematopoiesis in the spleen was noted at the highest dose (males, 55%; females, 72%, versus 43% and 57% in controls respectively). This finding was consistent with effects on erythrocytes seen in shorter studies, but was not statistically significant and was only slightly above the high background rate in controls.

The incidence of bronchiolo-alveolar adenoma or carcinoma was slightly increased in females at 2800 and 7000 mg/kg (10% for each group) compared with controls, and 70 mg/kg (3.3% for each group) (Table 16). There was no clear evidence of an increased incidence in males. The trend in females was statistically significant, but no pair-wise comparison was statistically significant. The incidence of these adenomas (3–8%) and carcinomas (3–7%) in females at 2800 and 7000 mg/kg was within the range for historical controls for adenomas (up to 15%) and carcinomas (up to 10%) reported for female Charles River

Table 16. Incidence of lung tumours in mice given diets containing methoxyfenozide for 18 months

Lung tumour	Dietary concentration (mg/kg)			
	0	70	2800	7000
<i>Males</i>				
Lung, adenoma, bronchiolo-alveolar	8/60	6/60	10/60	8/60
Lung, carcinoma, bronchiolo-alveolar	1/60	2/60	3/60	1/60
Lung, adenoma or carcinoma, bronchiolo-alveolar	9/60	8/60	13/60	8/60
<i>Females</i>				
Lung, adenoma, bronchiolo-alveolar	2/60	0/60	2/60	5/60
Lung, carcinoma, bronchiolo-alveolar	0/60	2/60	4/60	1/60
Lung, adenoma or carcinoma, bronchiolo-alveolar	2/60	2/60	6/60	6/60

From Robinson et al. (1998)

mice of this strain (Lang, 1995; no data were available from the test facility). Overall, it was considered that the lung tumours were not treatment-related.

The NOAEL for carcinogenicity and non-neoplastic effects was 7000 mg/kg (equal to 1020 mg/kg per day), the highest dose tested (Robinson et al., 1998).

Rats

Groups of 70 male and 70 female Sprague-Dawley rats were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0 (control), 200, 8000, or 20 000 mg/kg. These concentrations resulted in mean intakes of 0, 10.2, 411 and 1045 mg/kg per day in males, and 0, 11.9, 491 and 1248 mg/kg per day in females. The study complied with GLP and OECD guideline 453. Rats had access to food ad libitum and were housed individually. Satisfactory stability homogeneity and content of the test material were demonstrated. All rats were observed daily for signs of ill health or reaction to treatment. Physical examinations were performed weekly. Body weight and food consumption were monitored weekly for the first 13 weeks of dosing and once every fourth week thereafter. Ophthalmoscopic examinations were performed on all rats during the period before the start of dosing, and on all surviving rats before the termination of treatment. Urine analysis parameters were evaluated for at least 10 rats of each sex per group at 3, 6, 12 and 18 months of treatment, and again before the termination of treatment. Haematology (including methaemoglobin from 6 months) and clinical chemistry parameters were measured from 20 rats of each sex per group at 3, 6, 12 and 18 months (there was no information on fasting status). At the time of the terminal necropsies, blood samples were collected and analysed from 20 rats of each sex per group, or from all surviving animals when less than 20 animals remained in a group. The same animals were bled on each occasion when possible. White blood cell differential count was conducted for controls and animals at the highest dose only. At 12 months, 10 rats of each sex per group were necropsied and organ weights were recorded. All rats surviving to termination were necropsied and organ weights recorded. An extensive histopathological examination was performed for all controls and animals at the highest dose at sacrifice at 12 months and at termination, and on all animals not surviving to termination. At scheduled necropsy of animals at the lowest and intermediate doses, histopathological examination was limited to kidneys, liver, lungs, gross lesions and any tissues with potentially treatment-related lesions in animals at the highest dose. Mammary glands and pituitary glands of all females were examined histopathologically. An independent peer review by a second pathologist confirmed the histopathological findings.

Survival was poor in all groups, but overall this was considered not to have a significant impact on the interpretation of the results. There was an indication that time to 50% mortality was reduced at 20 000 mg/kg (Table 17). Less than 50% survival at 2 years (as recommended in the OECD guideline 453) is a recognized problem with the strain of rat used in this study (Charles River Sprague-Dawley rats, Crl:CD®BR).

No substance-related clinical signs of toxicity were noted at any concentration. Body weight was decreased in females at 20 000 mg/kg from week 45 onwards, which was statistically significant ($p < 0.05$) from week 73. Cumulative body-weight gain in males at the highest dose was reduced by 2.5% at week 53 and by 6% at termination and was not considered to be an adverse effect. There were no effects on food consumption.

Statistically significant, minimal to slight haematological changes were observed at 8000 mg/kg and 20 000 mg/kg in both sexes (Table 18). At 8000 mg/kg, erythrocyte count was decreased in both sexes, and haemoglobin concentration and erythrocyte volume fraction were decreased in males at week 14. At 20 000 mg/kg, erythrocyte counts, haemoglobin, and erythrocyte volume fraction were decreased during the first year of treatment. A slight increase in platelets and a minimal increase in methaemoglobin formation was seen at 20 000 mg/kg during the second year of treatment. The only clinical chemistry finding of note was an increase in plasma γ -glutamyl transferase at ≥ 8000 mg/kg, consistent with the observed hepatotoxicity. Statistically significant decreases in bilirubin and alkaline phosphatase were seen at ≥ 8000 mg/kg, but were not considered to be of toxicological importance. There were no consistent effects on any urine analysis parameters. Ophthalmoscopic examination at termination of treatment indicated there were no ocular lesions associated with treatment.

Increases in relative and absolute liver weights were seen at ≥ 8000 mg/kg in both sexes, with an increase in female adrenal weights at 20 000 mg/kg (Table 19). No treatment-related gross changes were seen. Primary target organs were kidney (glomerular nephropathy), liver (hepatocellular hypertrophy) and thyroid (follicular hyperplasia) (Tables 20 and 21). Effects were seen consistently at ≥ 8000 mg/kg. An increase in the severity of glomerular nephropathy at 200 mg/kg did not fit the dose-response relationship and was considered to be incidental. There were also several substance-related pathological changes in females at the highest dose, which the study investigators considered were secondary to uraemia

Table 17. Survival data for rats given diets containing methoxyfenozide in a study of chronic toxicity and carcinogenicity

Dietary concentration (mg/kg)	Weeks to 50% survival	Duration of treatment at terminal necropsy	Survivors at necropsy	Survival (%)
<i>Males</i>				
0	88–89	99	16	27
200	85	99	18	30
8 000	85–86	99	20	33
20 000	82	89	17	28
<i>Females</i>				
0	89–90	95	18	30
200	90	95	22	37
8 000	83	95	16	27
20 000	80	95	19	32

From Anderson & Gillette (1998)

Table 18. Haematology findings in rats given diets containing methoxyfenozide for up to 99 weeks

Parameter ^a	Dietary concentration (mg/kg)			
	0	200	8000	20 000
<i>Males</i>				
Erythrocyte count (10 ⁶ /μl)				
Week 14	7.67	7.57	7.16*	6.96*
Week 54	8.53	8.19	8.28	7.93*
Week 78	7.38	7.89	7.79	7.14
Termination	7.42	7.02	7.21	6.99
Haemoglobin (g/dl)				
Week 14	14.5	14.3	13.6*	13.3*
Week 54	15.4	14.9	15.2	14.7*
Week 78	14.1	14.6	14.2	13.8
Termination	14.2	13.6	13.4	13.3
Erythrocyte volume fraction (l/l)				
Week 14	0.442	0.438	0.414*	0.406*
Week 54	0.495	0.480	0.484	0.467*
Week 78	0.441	0.465	0.451	0.427
Termination	0.440	0.420	0.420	0.413
Platelet (10 ³ /μl)				
Week 14	960	961	993	943
Week 54	913	1005	941	930
Week 78	930	1039	987	1082
Termination	1112	1258	1210	1270
Methaemoglobin (%)				
Week 14	ND	ND	ND	ND
Week 54	0.7	0.6	0.6	0.9*
Week 78	1.2	0.8	1.1	1.2
Termination	0.7	0.7	0.9	0.9*
<i>Females</i>				
Erythrocyte count (10 ⁶ /μl)				
Week 14	6.91	6.86	6.57*	6.33*
Week 54	7.52	7.36	7.40	7.07*
Week 78	7.03	7.01	6.73	6.68
Termination	6.84	6.77	6.73	6.96
Haemoglobin (g/dl)				
Week 14	14.2	13.9	13.6	13.1*
Week 54	15.4	15.0	15.1	14.3*
Week 78	14.7	14.6	14.3	13.9
Termination	14.1	14.1	13.8	14.1
Erythrocyte volume fraction (l/l)				
Week 14	0.433	0.432	0.417	0.405*
Week 54	0.480	0.469	0.475	0.453
Week 78	0.447	0.444	0.432	0.422
Termination	0.429	0.431	0.423	0.432
Platelets (10 ³ /μl)				
Week 14	897	903	904	948
Week 54	829	893	844	970*
Week 78	836	853	869	961*
Termination	926	1020	948	1027
Methaemoglobin (%)				
Week 14	ND	ND	ND	ND
Week 54	0.6	0.5	0.6	0.8
Week 78	1.2	0.9*	1.1	1.2
Terminal	0.8	0.8	0.8	1.1*

From Anderson & Gillette (1998)

^a Means of 20 animals per dose per time-point, except at termination, when there were 15–20 animals per dose

* Significant difference from control ($p < 0.05$); ANOVA, Dunnett t -test

ND, Not determined

Table 19. Mean liver and adrenal weights in rats given diets containing methoxyfenozide for up to 99 weeks

		Dietary concentration (mg/kg)							
		0		200		8000		20000	
		12 months	Termination	12 months	Termination	12 months	Termination	12 months	Termination
<i>Males</i>									
Liver									
Absolute weight (g)		22.9	18.9	26.1	20.0	27.6	19.9	32.2*	23.2*
Relative weight (%)		3.1	2.6	3.3	2.5	3.7*	2.8	4.1*	3.05
Adrenals									
Absolute weight (g)		0.06	0.08	0.06	0.08	0.08*	0.12	0.07	0.10
Relative weight (%)		0.008	0.011	0.008	0.010	0.010	0.017	0.009	0.013
<i>Females</i>									
Liver									
Absolute weight (g)		15.9	16.4	15.1	14.9	16.4	17.5	17.7	16.6
Relative weight (%)		3.3	2.6	3.3	2.7	3.6	2.9	3.8*	3.3*
Adrenals									
Absolute weight (g)		0.08	0.10	0.07	0.14	0.08	0.12	0.12*	0.15
Relative weight (%)		0.017	0.016	0.016	0.025	0.017	0.021	0.025*	0.032*

From Anderson & Gillette (1998)
* Significant difference from control (*p* < 0.05); ANOVA, Dunnett *t*-test

that occurred as a result of severe chronic progressive glomerulonephropathy. These included increases in mineralization of the heart, aorta and structures in the kidney; fibrous osteodystrophy of the bone. Acute to subacute inflammation and erosion/ulceration of the forestomach and mineralization and associated giant cell inflammation of the glandular stomach were also increased at 8000mg/kg (Table 20).

There was a slightly increased incidence of hepatocellular adenoma in females at the highest dose (Table 21). The incidence in this group (5.7% of all females, 6.7% of females at final sacrifice plus early deaths) is consistent with ranges for historical controls for hepatocellular adenoma in this strain of Charles River rat (females, 0.8–13.3%, males, 1.4–8.0% (Giknis & Clifford, 2001); females, 1.4–21.7%, males, 0–16.7% (McMartin et al., 1992). These historic control data are not perfect as they are from a range of test facilities and are not specific to the laboratory that performed the main study. The adenomas observed in this study were reported to be of similar appearance and size to that of spontaneous tumours. There was no increase in mitotic figures, the incidence/severity of altered foci or hepatocellular carcinoma. There was no increase in the incidence of hepatocellular tumours in males. Overall, it is considered that although there was marked liver hypertrophy in females at the highest dose (Table 20), there was no clear substance-related increase in the incidence of hepatic tumours in this study. The increased incidence (14.3%) of thyroid C-cell adenoma in males at 8000mg/kg bw (Table 21) was not part of a dose–response relationship and was just within the range of incidence for historic controls reported for males of this strain by Giknis & Clifford, 2001 (1.4–14.3%), but slightly exceeded that reported by McMartin et al., 1992 (2.9–8.7%). Overall, it was considered that there was no substance-related increase in the incidence of thyroid tumours in this study. Statistically significant increased incidences of mammary gland adenoma were seen in females at 200 and 8000mg/kg bw (Table 21). This is, however, not considered to be a substance-related effect because of the lack of a dose–response relationship across groups that showed similar survival to termination. It is also notable that on the basis of the number of females with mammary gland adenocar-

Table 20. Histopathology findings in rats given diets containing methoxyfenozide for up to 99 weeks

Finding	Dietary concentration (mg/kg)			
	0	200	8000	20000
At sacrifice at 12 months:				
<i>Males</i>				
Chronic progressive glomerulonephropathy				
Minimal	6/10	2/10	6/10	2/10
Slight	0/10	3/10	4/10	0/10
Moderate/moderately severe	1/10	3/10	0/10	8/10
Total incidence	7/10	8/10	10/10	10/10
Periportal hepatocellular hypertrophy	0/10	0/10	8/10	10/10
Thyroid hypertrophy, follicular	2/10	0/10	4/10	1/10
Thyroid, altered colloid	1/10	1/10	4/10	3/10
<i>Females</i>				
Chronic progressive glomerulonephropathy	5/10	2/10	4/10	8/10
Periportal hepatocellular hypertrophy	0/10	0/10	6/10	8/10
Thyroid hypertrophy, follicular	1/10	0/10	4/10	5/10
Thyroid, altered colloid	1/10	0/10	0/10	4/10
All animals:				
<i>Males</i>				
Kidney, hyperplasia, epithelial, pelvis	10/70	9/70	5/70	15/70
Periportal hepatocellular hypertrophy	1/70	0/70	29/70***	42/70***
Thyroid hypertrophy, follicular	10/70	5/68	19/70*	19/69*
Thyroid, altered colloid	8/70	11/68	17/70*	17/69*
<i>Females</i>				
Chronic progressive glomerulonephropathy				
Moderate to severe	8/70	7/70	6/70	14/70
Total incidence	51/70	50/70	53/70	60/70*
Kidney, hyperplasia, epithelial, pelvis	10/70	17/70	14/70	23/70**
Periportal hepatocellular hypertrophy (minimal to moderate)	2/70	2/70	30/70***	50/70***
Thyroid hypertrophy, follicular	10/70	4/70	14/70	22/70*
Thyroid, altered colloid	11/70	3/70	11/70	27/70**
Aorta, mineralization	0/70	2/70	1/70	6/69*
Heart, mineralization	0/70	2/70	1/70	5/70*
Kidney, mineralization	0/70	2/70	0/70	6/70*
Bone, fibrous osteodystrophy	0/70	2/70	1/70	5/70*
Forestomach, inflammation	2/70	2/70	5/70	9/70*
Forestomach, oedema	2/70	3/70	6/70	8/70*
Forestomach, erosion/ulceration	2/70	2/70	4/70	7/70*
Glandular stomach, mineralization	0/70	2/70	1/70	6/70*
Glandular stomach, inflammation, giant cell	0/70	1/70	1/70	5/70*

From Anderson & Gillette (1998)
p* < 0.05; *p* < 0.01; ****p* < 0.001

cinoma, or the related adenoma and fibroadenoma, none of the test groups showed an increased incidence compared with that in the negative control group (Table 21). The incidence of mammary gland adenocarcinoma in females at 200 and 8000 mg/kg bw (23–25% of all females) was well within the historical ranges for females of this strain—6.7–30% (McMartin et al., 1992) and 8.6–58.3% (Giknis & Clifford, 2001).

Within the limitations of the study (reduced survival, generic historic control data) it is considered that there was no clear oncogenic response at doses of up to 20000 mg/kg (equal to 1045 mg/kg bw per day). The overall NOAEL was 200 mg/kg (equal to 10 mg/kg bw per day) on the basis of reductions in erythrocyte parameters, increased incidence of histopathological changes in the liver (periportal hepatocyte hypertrophy) and thyroid

Table 21. Incidence of neoplastic histopathology in rats given diets containing methoxyfenozide for up to 99 weeks (animals surviving to termination)

Finding	Dietary concentration (mg/kg)			
	0	200	8000	20 000
<i>Males</i>				
Liver				
Hepatocellular adenoma	0/70	0/70	3/70	2/70
Hepatocellular carcinoma	3/70	0/70	2/70	1/70
Thyroid				
C-cell adenoma	2/70 (1/16)	2/68 (1/18)	10/70 ^a (7/20)	4/69 (2/17)
C-cell carcinoma	0/70	0/68	0/70	0/69
<i>Females</i>				
Liver				
Hepatocellular adenoma	0/70 (0/18)	1/70 (1/22)	2/70 (2/16)	4/70 ^b (3/19)
Hepatocellular carcinoma	0/70	0/70	0/70	0/70
Thyroid				
C-cell adenoma	2/70	2/70	4/70	4/70
C-cell carcinoma	0/70	1/70	0/70	0/70
Mammary gland				
Adenoma	3/68	4/69	3/69	3/68
Fibroadenoma	28/68	24/69	25/69	19/68
Adenocarcinoma	6/68 (0/18)	17/69 ^c (1/21)	16/69 ^d (0/15)	8/68 (1/19)
Sum of these three tumours	33/68	38/69	36/69	25/68

From Anderson & Gillette (1998)
^a*p* = 0.0168 (survival-adjusted analysis)
^b*p* = 0.0275 (survival-adjusted analysis)
^c*p* = 0.0337 (survival-adjusted analysis)
^d*p* = 0.0156 (survival-adjusted analysis)

(follicular hypertrophy and altered colloid) at 8000 mg/kg (equal to 411 mg /kg bw per day) (Anderson & Gillette, 1998).

Dogs

No long-term studies in dogs were submitted.

2.4 Genotoxicity

Methoxyfenozide has been investigated for its ability to induce gene mutations in bacteria and mammalian cells in vitro, for the induction of chromosomal aberrations in mammalian cells and in an assay for micronucleus formation in mice in vivo (Table 22). Precipitation of methoxyfenozide limited the maximum concentration that could be used. All studies gave negative results, and complied with the OECD guideline current at the time of performance and with GLP. A repeat Ames test was performed because the original test did not use strains (TA102 or *E. coli* WP2) regarded as being sensitive to hydrazines (methoxyfenozide is a hydrazine derivative, as are some impurities of methoxyfenozide). The purity of the material tested was greater than that of the proposed technical specification, but the impurity profile (qualitative and quantitative) of the technical material (97%) did not give rise to any significant concerns regarding genotoxicity. The results of an Ames test and assay for micronucleus formation in vivo with a flowable formulation (240SC) were negative. The Meeting concluded that methoxyfenozide (technical material) was unlikely to be genotoxic.

Table 22. Results of studies of genotoxicity with methoxyfenozide

End-point	Test object	Concentration/dose	Purity (%)	Results	Reference
<i>In vitro</i>					
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537	50–5000 µg/plate ± S9	98	Negative +S9, negative –S9	Sames & Streelman (1995)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102, TA1535, TA1537	50–5000 µg/plate ± S9	99.2	Negative +S9, negative –S9	Sames & Ciaccio (1998)
Reverse mutation	<i>S. typhimurium</i> TA98 ^a , TA100, TA102, TA1535, TA1537	50–5000 µg/plate + S9	23.3; formulation 2F 240SC	Negative +S9, negative –S9	Sames & Ciaccio (1996)
Gene mutation	Chinese hamster ovary cells, <i>Hprt</i> locus	0.5–100 µg/ml ± S9 ^b	99.2	Negative +S9, negative –S9	Pant (1994)
Chromosome aberrations	Chinese hamster ovary cells	13–150 µg/ml ± S9 ^c (multiple exposure and sample times)	99.2	Negative +S9, negative –S9	Kumaroo (1994)
<i>In vivo</i>					
Micronucleus formation	CD-1 mice (five of each sex per group), bone-marrow cells	0, 500, 2500, 5000 mg/kg bw, by gavage, in methylcellulose	99	Negative	Sames & Black (1995)
Micronucleus formation	CD-1 mice (five of each sex per group), bone-marrow cells	0, 1250, 2500, 5000 mg/kg bw, by gavage, in methylcellulose	23.6; formulation 2F	Negative	Sames & Ciaccio (1997)

S9, 9000 × *g* supernatant fraction of rodent liver

^aPerformed as a separate assay, owing to contamination

^bPrecipitation at 100 µg/ml; considerable variation in mutation rates between duplicate culture

^cPrecipitation and reduced mitotic index at 150 µg/ml

2.5 Reproductive toxicity

(a) Multigeneration study

In a two-generation study that complied with GLP and OECD test guideline 416 (1983) and also included some additional investigations (i.e. sperm tests, estrous cycling and developmental milestones), groups of CrI:CD®BR rats (one litter per generation) were given diets containing methoxyfenozide (purity, 98.0%) at a concentration of 0, 200, 2000, or 20 000 mg/kg active substance. Satisfactory stability, content and homogeneity were demonstrated. Mean intake of methoxyfenozide is shown in Table 23. Doses were selected on the basis of the findings of the 90-day dietary study in rats.

Exposure of P₁ animals (30 of each sex per dose; not littermates) began when the animals were aged approximately 6 weeks. P₂ animals (30 of each sex per dose) were randomly selected from P₁ offspring (designated F₁) and given treated diet from weaning. P₁ and P₂ animals were mated after at least 10 weeks of exposure.

Body weight, food consumption, and clinical signs were monitored in parental animals throughout treatment. Estrus cycling was evaluated in females for 3 weeks before mating. Parental animals were necropsied after weaning of their offspring. Sperm evaluation was performed for all P₁ and P₂ males at the time of necropsy. Weights of the liver and organs of the reproductive system were determined in parental animals. Reproductive tissues, pituitary and liver were examined histopathologically in all control animals and animals at the highest dose, and in all animals found dead or sacrificed during the course of the study. Tissues in which treatment-related lesions were identified were also examined in all animals

Table 23. Mean intake of methoxyfenozide in a two-generation study in rats

	Intake (mg/kg bw per day)		
	200	2000	20 000
<i>Males</i>			
P ₁ pre mating	15.4	153.4	1 551.9
P ₂ pre mating	19.1	193.1	1 956.0
<i>Females</i>			
P ₁ pre mating	17.9	180.7	1 820.7
P ₂ pre mating	20.4	202.7	2 036.5
P ₁ gestation	14.7	147.9	1 563.7
P ₂ gestation	14.1	143.0	1 474.0
P ₁ lactation	26.7	263.6	2 657.4
P ₂ lactation	26.1	268.7	2 612.6

From O'Hara et al. (1997)

at the lowest and intermediate doses. In addition, reproductive tissues were examined in animals at the lowest and intermediate doses that were suspected to have reduced fertility. All tissues with gross pathological changes were examined microscopically. Litters were culled to eight pups (four of each sex per litter, where possible) on day 4 of lactation. Still-born pups, pups that died during lactation, pups culled at day 4, or sacrificed at weaning were examined grossly. Sexual maturation (age at preputial separation in males, vaginal patency in females) was evaluated in F₁ offspring selected as P₂ parental animals, and F₂ females (one per litter). Anogenital distance was measured in all F₂ offspring on the day of birth.

There were no substance-related deaths or clinical signs of systemic toxicity in parental animals or in pups during the lactation period. Decreased body-weight gain was observed at 20 000 mg/kg in P₁ males (about 10%) and to a lesser extent in P₂ males (about 3%), beginning at week 8 of treatment and continuing throughout the remaining treatment period. There were no adverse substance-related effects on female body weight. Food consumption was similar in all groups, statistically significant increased feed consumption during gestation (by up to 12–16% in the P₁ animals at the highest dose) were linked to increased body weights, showed no clear dose-related trend and are considered to be incidental findings.

There were no substance-related effects on estrous cycling, sperm parameters, mating performance, litter size, pup body weight or pup viability. An apparent increase in the number of stillborn F₁ pups was reversed for the F₂ generation and was not considered to be an adverse effect of treatment (Table 24). There was no consistent effect on pup sex ratio. A statistically significant reduction in the proportion of male pups in the F₂ offspring at day 0 after exposure to methoxyfenozide at 20 000 mg/kg was considered to be incidental. Although the proportion of F₂ male pups at 20 000 mg/kg (44.7%) was slightly below the laboratory's lowest range for historical controls (47.7–53.4%), the statistical results were skewed as the control value (56.5%) was also outside the range for historical controls (Table 24). In addition, there was no evidence of a male-specific effect in utero (no decrease in the number of pups delivered per litter) and no evidence of feminization of male offspring, as determined by anogenital distance (Table 24). There were no adverse gross findings in F₁

Table 24. Litter findings (means) in rats given diets containing methoxyfenozide in a two-generation study

Parameter	Intake (mg/kg of feed)			
	0	200	2000	20 000
P ₁ pups stillborn	0/259 (0%) ^b	2/312 (0.6%)	2/373 (0.5%)	7/320* (2.2%)
P ₂ pups stillborn	8/311 (2.6%)	2/381* (0.5%)	3/360 (0.8%)	2/367* (0.5%)
P ₁ litter size	11.8	13.6	13.8	13.9
P ₂ litter size	13.0	13.6	15.0	13.6
F ₁ , % male pups				
Day 0	46.3%	52.3%	52.2%	48.3%
Day 21	46.9%	48.6%	49.6%	48.5%
F ₂ , % male pups				
Day 0	56.5%	50.2%	52.5%	44.7%*
Day 21	55.3%	48.5%	50.3%	49.0%
F ₁ pup weight, day 21 (g)	59.7	60.2	58.3	59.5
F ₂ pup weight, day 21 (g)	58.1	61.0	61.4	58.3
F ₁ , days to vaginal patency (<i>n</i> = 22–25)	30.9 ± 0.42 (range, 28–35)	32.0 ± 0.42	31.6 ± 0.42	34.0* ± 0.55 (range, 29–42)
F ₂ , days to vaginal patency (<i>n</i> = 21–28)	31.8 ± 0.39 (range, 29–35)	31.9 ± 0.51	31.6 ± 0.43	33.3 ± 0.54 (range, 29–39)
Body weight on day of vaginal patency (g) ^a	125.0	125.3	124.6	128.3
F ₂ female pups, day 0, (<i>n</i> = 22–28)				
Anogenital distance (mm)	1.06	1.11	1.13*	1.10
F ₂ male pups, day 0, (<i>n</i> = 24–28)				
Anogenital distance (mm)	2.50	2.51	2.55	2.53

From O'Hara et al., (1997)

**p* < 0.05 (Fisher's exact test, Mann-Whitney U test or ANOVA with Dunnett *t*-test)

^aTest laboratory's historical control data for the same strain of rat (five studies for 1995–2000): mean days to vaginal patency = 31.3–32.6 days; mean weight at day of vaginal patency = 111–128 g

or F₂ offspring at any dietary concentration. There were no substance-related effects on sexual maturation in F₁ males (age at preputial separation) at any dietary concentration.

A slight delay in attainment of vaginal patency was noted in F₁ females (statistically significant) and in F₂ females (not statistically significant) at 20 000 mg/kg (Table 24), the values being outside the range for historical data for the test facility. There was no indication that this might be linked with outliers or be secondary to reduced body weight or general pup development. However, there was no effect on mating or reproductive success subsequent to this delay. There were no specific effects on the weight of the reproductive organs examined; increases in relative testes and epididymal weights were secondary to reduced body weights. There were no pathological changes in the reproductive organs examined. A significant increase in absolute and relative liver weight (13–27%), increased incidences of hepatocellular hypertrophy (periportal or midzonal) and pigmentation of Kupffer cells were seen in parental animals exposed at 20 000 mg/kg. A statistically significant but slight (7%) increase in relative liver weight seen in P₁ males and mild hepatocellular hypertrophy in four out of 30 P₁ females at 2000 mg/kg were not considered to be adverse effects.

The minimal effects on the liver at 2000 mg/kg in parental (P₁) animals at 2000 mg/kg was not considered to be an adverse effect. The NOAEL for general systemic toxicity was therefore 2000 mg/kg (equal to 143 mg/kg bw per day) on the basis of reduced body-weight gain, increased liver weight (≥10%) and histopathological changes in liver (hepatocellular hypertrophy and vacuolation, pigment in Kupffer cells) at 20 000 mg/kg.

The NOAEL for reproductive outcome was 20 000 mg/kg (equal to 1474 mg/kg bw per day), the highest dose tested.

The NOAEL for pup development was 2000 mg/kg (equal to 143 mg/kg bw per day) on the basis of a slight delay in attainment of vaginal patency in pups at the highest dose that was seen in both generations, with no subsequent effect on reproduction or general development (O'Hara et al., 1997).

(b) *Developmental toxicity*

Rats

Groups of 25 mated female Crl:CD BR Sprague-Dawley rats were given methoxyfenozide (purity, 99.2%) at a dose of 0, 100, 300 or 1000 mg/kg bw per day on days 6–15 of gestation (day 0 = plug observed) by gavage in aqueous carboxymethylcellulose. Dams were sacrificed on day 20 of gestation and fetuses were examined. Approximately half of the fetuses in each litter were examined for visceral abnormalities by serial sectioning, the remainder were examined for skeletal abnormalities after staining with alizarin red. This study complied with GLP and OECD guideline 414 (1981).

No animals died and no clinical signs of toxicity were observed. No treatment-related effects were seen on maternal body weights, body-weight gain or food consumption. Gross necropsy of dams did not reveal any treatment-related findings. No treatment-related effects were seen on reproductive or litter parameters (including percentage of live male fetuses). Total incidences of fetal alterations were significantly lower in treated groups. Ossification of a number of bones was found to be greater in treated groups (Table 25). The finding of

Table 25. Developmental findings in rats given methoxyfenozide by gavage during organogenesis

Parameter	Dose (mg/kg bw per day)							
	0		100		300		1000	
<i>Reproductive parameters</i>								
No. pregnant	25		25		22		24	
Corpora lutea	18.1		17.7		17.5		17.4	
Implantations	15.1		15.1		14.3		14.9	
Number of litters	25		25		22		24	
<i>Litter parameters</i>								
Litter size	14.7		14.7		13.6		14.5	
Fetal viability	100%		100%		100%		100%	
Fetal weight	3.33		3.38		3.36		3.36	
Live male fetuses/litter	54.8%		47.4%		54.7%		49.5%	
<i>Fetal parameters: % litter incidence (% fetal incidence)</i>								
Fetuses with alteration (total)	44.0	(6.8)	44.0	(4.9)	18.2	(1.3**)	20.8	(3.2**)
↓Ossification, lumbar arch	16.0	(2.2)	0.0**	(0.0**)	4.5**	(0.6**)	0.0**	(0.0**)
↓Ossification, sternbrae	12.0	(3.8)	8.0	(1.0**)	0.0	(0.0**)	4.2	(1.1**)
↓Ossification, pubes	28.0	(8.1)	28.0	(5.3)	9.1	(1.3**)	8.3	(2.2**)
↓Ossification, ischia	16.0	(2.7)	8.0	(2.6)	0.0	(0.0**)	0.0	(0.0**)
Bifid thoracic centra ^a	4.0	(0.5)	4.0	(0.5)	4.5	(0.6)	12.5	(1.6)

From Hoberman (1994)

↓, Decreased

** Significantly different to controls ($p < 0.01$)

^aMARTA and MTA (1996): historical control data: average litter incidence, 8% (maximum value, 75%); average fetal incidence, 1.2% (maximum value, 10.7%)

a single fetus with multiple abnormalities at the highest dose was not considered to be a treatment-related finding. The incidence of bifid thoracic vertebral centra was marginally increased at the highest dose; however, values did not attain statistical significance (Table 25). The litter and fetal incidence for this lesion at the highest dose were very similar to means for historical controls reported for this strain of rat by MARTA and MTA databases (1996), (Table 25), and it was considered that the marginally increased incidence of bifid thoracic centra at the highest dose in the present study was not of clear toxicological significance.

No evidence of teratogenicity was seen. The NOAEL for maternal toxicity was 1000mg/kgbw per day on the basis of the absence of toxicity at the highest dose of 1000mg/kgbw per day (limit dose). The NOAEL for developmental toxicity was 1000mg/kgbw per day (Hoberman, 1994).

Rabbits

Groups of 16 mated female New Zealand white rabbits were given methoxyfenozide (purity, 98%) at a dose of 0, 100, 300, or 1000mg/kgbw per day on days 7–19 of gestation (day 0 = day of mating) in aqueous carboxymethylcellulose by gavage. Dams were sacrificed on day 29 of gestation and fetuses were examined after caesarean section. All fetuses were examined for visceral abnormalities and for skeletal abnormalities after staining with alizarin red. This study complied with OECD guideline 414 (1981) and with GLP.

One control animal died after an intubation error and one animal at 300mg/kgbw per day aborted on day 22 of gestation. No clinical signs of toxicity were noted. Mean body-weight gain of animals at the highest dose was decreased during days 20–29, but this was seen as a correction for increased body-weight gain during the dosing period (Table 26). No treatment-related gross lesions were detected on examination of the does post mortem. No treatment-related effects on reproductive or litter parameters were seen. The slightly lower mean litter size at the highest dose reflects the lower numbers of corpora lutea and implantation sites and was not treatment related. A statistically significant decrease in the number of live male fetuses was seen at 1000mg/kgbw per day; however, this finding was not considered to be substance-related because the value was within the range for normal variation and the control group had an unusually high number and percentage of male fetuses compared with historical controls (Table 26). No substance-related skeletal malformations were noted.

Skeletal retardations were slightly decreased at the highest dose owing to the increased ossification of sternebrae and vertebrae; this finding was not considered to constitute an adverse effect. The total fetal incidence of soft tissue findings was slightly increased at the highest dose, largely due to the increased incidence of a circulatory system alteration (left carotid artery arising from the innominate artery). This finding is classified as a developmental variation. When incidences are expressed as a percentage (Table 26), there appears to be a dose-related increase, at all doses, in the fetal incidence and litter incidence of this variation. Although the number of fetuses with the finding increased from one in the controls to 11 at the highest dose, the number of litters involved was small (increasing from only one in the controls to three at the highest dose), incidences were within the range for historical controls, and analysis of the litter incidence revealed no statistically significant differences ($p > 0.05$ in Fisher exact test). It was considered that the apparent increased

Table 26. Summary of findings in a study of developmental toxicity in rabbits

Parameter	Dose (mg/kg bw per day)							
	0		100		300		1000	
<i>Reproductive parameters</i>								
Weight gain (days 0–29)	280.3		364.7		317.4		292.4	
Weight gain (days 7–20)	143.1		159.6		146.6		193.5	
Weight gain (days 20–29)	184.9		226.6		188.2		150.8	
No. pregnant	16		16		15		15	
No. of litters	15		16		14		15	
<i>Litter parameters</i>								
Corpora lutea	10.4		10.9		10.3		9.8	
Implantation sites	9.7		10.0		9.9		8.8	
Litter size	9.5		9.4		9.9		8.7	
Fetal viability (%)	100		100		100		100	
Litter weight (g)	40.1		43.1		39.0		42.7	
Live females/litter	3.5		4.4		4.7		4.5	
Live males/litter ²	6.1		5.1		5.1		4.2*	
% live males/litter ³ (%)	63.6		52.1		52.1		49.5	
<i>Fetal findings: % fetal incidence and [% litter incidence]</i>								
Total soft tissue observations	4.9	[33.3]	6.6	[50.0]	4.3	[21.4]	10.7	[33.3]
Left carotid variation ¹	0.7	[6.7]	2.0	[12.5]	3.6	[14.2]	8.4	[20.0]
	(1) ^a	(1) ^b	(3) ^a	(2) ^b	(5) ^a	(2) ^b	(11) ^a	(3) ^b
Total skeletal retardations	70.6	[100]	66.2	[100]	72.5	[92.9]	63.4	[93.3]
Unossified sternebrae	16.1	[53.3]	9.3	[43.8]	7.2	[35.7]	9.9	[33.3]
Partially ossified arches of lumbar vertebrae	43.4	[93.3]	36.5	[75.0]	42.8	[92.9]	35.1	[80.0]

From Shuey (1997)
**p* < 0.05, ^a = fetuses, ^b = litters; with one or more fetuses with left carotid arising from innominate
Historical control range data for the test laboratory
¹ Left carotid artery arising from innominate: 3.3–9.0% (fetal incidence); 13–46% (litter incidence)
² Number of live males/litter (1990–1996): 4.1–4.7 (litter incidence)
³ Live males/litter (1990–1996): 50.3–57.8% (litter incidence)

incidence of this variation in the present study was not a toxicologically significant adverse finding.

No evidence of teratogenicity was seen. The NOAEL for maternal toxicity was 1000mg/kgbw per day on the basis of the absence of any toxicologically significant effects at the limit dose. The NOAEL for developmental toxicity was 1000mg/kgbw per day on the basis of the absence of clear substance-related adverse findings at the highest dose of 1000mg/kgbw per day (limit dose) (Shuey, 1997).

2.6 Special studies

(a) Acute neurotoxicity in rats

In a study that complied with GLP and that was according to US EPA guidelines, groups of 10 male and 10 female Crl:CD®BR rats (aged about 6 weeks) were given a single dose of methoxyfenozide (purity, 98%) at 0, 500, 1000, or 2000mg/kgbw by gavage suspended in 0.5% aqueous methylcellulose at a constant volume of 20ml/kg. Analysis of suspensions showed satisfactory concentrations of active substance. All rats were observed daily for signs of ill health or reaction to treatment and were weighed weekly. A functional observational battery (FOB) was performed on each rat pre-test and motor activity assessment was performed 7 days before dosing. FOB and motor activity testing was repeated within 3 h of dosing on day 0 and on days 7 and 14 after dosing. The time of testing on day

0 was chosen to be as close as feasible to the time of peak blood concentrations of active substance and/or metabolites. On day 14, after the final FOB and motor activity assessment, rats were perfused with neutral buffered formalin and given a limited gross necropsy. Twelve randomly selected control animals (six males and six females) and 12 randomly selected animals at the highest dose (six males and six females) received a special neuropathology evaluation that included examination of the brain, spinal cord, selected ganglia, spinal root fibres and peripheral nerves of the hindlimb. Stains used were haematoxylin and eosin, and toluidine blue. The study investigators reported that studies with six positive control substances conducted at the test laboratory in 1994 gave results consistent with the published literature.

No mortalities, substance-related clinical signs of systemic toxicity or body-weight effects were observed during the study period. There were no consistent substance-related effects on motor activity or any of the FOB parameters. Hind-limb grip strength for males at 2000 mg/kg bw was reduced statistically significantly on day 0 within 3 h of dosing; the reduction was by 24% (adjusted for pre-test mean); the corresponding mean hind-limb grip strength value for females at 2000 mg/kg bw showed a slight increase (13%). There was a statistically significant increase (by 31% after adjustment for the pre-test mean) in the total number of movements shown by males at 2000 mg/kg bw on day 14. Although slight increases were seen on days 0 and 7, they were not statistically significant, nor was the slight increase in total time spent by males in movement at 2000 mg/kg bw. In females at 2000 mg/kg bw, on day 14 the total number of movements decreased slightly (not statistically significantly) and there was no change in the total time spent in movement. Overall, there was considered to be no convincing evidence for effect on grip strength or motor activity.

Gross necropsy revealed no substance-related findings. No treatment-related histopathological alterations were observed in any of the examined areas of the central or peripheral nervous systems. A low incidence of minimal axonal degeneration, consisting of one to two focally degenerating axons, in peripheral nerves (maximum incidence, one out of six) was a consistent background finding. Methoxyfenozide, when administered orally by gavage as a single dose at doses up to and including 2000 mg/kg bw, produced no evidence for neurotoxic effects in rats. The NOAEL for neurotoxicity and neuropathology was 2000 mg/kg, the limit dose tested (Anderson & Gillette, 1996).

(b) Thirteen-week study of neurotoxicity in rats

In a study that complied with GLP and that was performed according to US EPA guidelines, groups 10 male and 10 female Crl:CD®BR rats (aged 6 weeks) were given diets containing methoxyfenozide (purity, 98%) at a concentration of 0 (control), 200, 2000, or 20 000 mg/kg for 13 weeks. These doses were equal to an average intake of 0, 13, 130, and 1318 mg/kg bw per day in males, and 0, 16, 159, 1577 mg/kg bw per day in females. Satisfactory stability, homogeneity and active substance content were demonstrated. All rats were observed daily for signs of ill health or reaction to treatment. Body weight and food consumption were monitored and physical examinations were performed weekly. Neurotoxicity testing, including FOB and motor activity assessments, were performed on all rats pre-test and on weeks 4, 8, and 13. All rats surviving to necropsy were perfused with neutral buffered formalin and given a limited gross necropsy. Histopathological evaluations of the nervous system were performed for six rats of each sex per group (at the highest dose and in the control group).

There were no substance-related mortalities or clinical signs of toxicity. The study investigators considered that there were no treatment-related effects on body weight. A slight, not statistically significant, reduction in overall body weight of 5–7% in both sexes at 20 000 mg/kg was not regarded as an adverse effect. No substance-related changes were seen in either the FOB or motor activity assessments.

There were no substance-related gross or microscopic pathological findings. Minimal axonal degeneration observed in peripheral nerves, consisting of one to two degenerating axons per nerve, was a typical background finding. There was no evidence of neurotoxic or neuropathic effects in rats receiving diets containing methoxyfenozide at concentrations of up to and including 20 000 mg/kg for 13 weeks. The NOAEL was 20 000 mg/kg (equal to 1318 mg/kg bw per day), the highest dose tested (Kane & Gillette, 1996).

(c) *Studies on metabolites*

The acute oral toxicity and mutagenicity of metabolite *N*-2–3-hydroxybenzoyl-*N'*-3,5-dimethylbenzoyl-*n*'-tert-butylhydrazine (M14) have been investigated. M14 was found to be of low acute oral toxicity in CD-1 (ICR) mice in a study that complied with GLP; no deaths were seen at 5000 mg/kg bw (Parno et al, 1997).

In a study that complied with GLP, no increases in mutation rates were seen in *S. typhimurium* strains TA98, TA100, TA102, TA1535 or TA1537 at concentrations up to the limit value of 5000 µg/plate, in the presence and absence of metabolic activation (Sames & Ciaccio, 1998).

3. Observations in humans

Commercialization of methoxyfenozide has only occurred recently and data on human exposures are limited. There have been no adverse health effects reported among manufacturing workers, users or the general population.

Comments

Orally administered [¹⁴C]methoxyfenozide is absorbed rapidly in rats; 58–77% of the administered dose was excreted within 24 h. Peak plasma concentrations of radioactivity (C_{\max}) were seen approximately 30 min after dosing. Excretion occurs mainly via the faeces, after absorption followed by secretion in the bile. On the basis of the quantities of radioactivity excreted in the bile and urine, approximately 60–70% of an orally administered dose of 10 mg/kg bw was absorbed. Absorption and excretion profiles were similar irrespective of dose (10 or 1000 mg/kg bw), single or repeated dosing (over 14 days) or sex, the only differences being evidence of saturation at the highest dose and a slightly increased level of urinary excretion in females. Concentrations of radioactivity at C_{\max} were highest in the liver, with concentration in the adrenals and in the spleen also being higher than that in whole blood.

More than 30 metabolites of methoxyfenozide were identified in rat urine, faeces and bile. The primary reactions were demethylation, glucuronidation and hydroxylation. Less than 5% of the methoxyfenozide administered was cleaved at the amide bridge between the two aromatic rings. Repeated dosing at 10 mg/kg bw for 14 days altered the metabolite profile to a limited extent, with an increase in the concentration of multiple hydroxylated compounds.

Methoxyfenozide has low acute toxicity when administered by the oral, dermal or inhalation routes. In rats, the acute LD₅₀ was > 5000 mg/kg bw after oral or dermal administration. Methoxyfenozide was not irritating to rabbit skin, and produced minimal transient irritation of the rabbit eye. Methoxyfenozide did not induce skin in a Magnusson and Kligman maximization test for sensitization in guinea-pigs.

Short-term studies in rats, mice and dogs fed with diets containing methoxyfenozide showed that these animals tolerated high concentrations of methoxyfenozide, equivalent to about 1000 mg/kg bw per day, with no marked adverse effects. Effects seen to varying degrees in all species were increased liver weight, hepatocyte hypertrophy and alterations in erythrocyte parameters consistent with a mild haemolytic effect, accompanied by formation of methaemoglobin. Findings were not always consistent between studies in the same species and comparison was also hindered to a certain extent by variations in blood sampling procedure and in the range of parameters investigated. The NOAEL in mice was 2500 mg/kg, equal to 428 mg/kg bw per day on the basis of reduced body-weight gain at 7000 mg/kg, equal to 1149 mg/kg bw per day, in the 90-day study. The NOAEL in rats was 1000 mg/kg, equal to 69 mg/kg bw per day, on the basis of increased (by >10%) relative liver weights and periportal hepatocyte hypertrophy at 5000 mg/kg, equal to 353 mg/kg bw per day, in the 90-day study. Thyroid follicular cell hypertrophy or hyperplasia seen in the 2-week study in rats receiving methoxyfenozide at 1000 mg/kg, equal to 98 mg/kg bw per day, was not reproduced in the 90-day study. In dogs, increases in the formation of methaemoglobin and abnormal erythrocyte morphology were seen in two 14-day studies, with increases in spleen weights also noted in one of these studies. The overall NOAEL in the 14-day studies in dogs was 500 mg/kg, equal to 20 mg/kg bw per day, with a lowest-observed-adverse-effect level (LOAEL) of 3500 mg/kg, equal to 154 mg/kg bw per day. No treatment-related adverse effects were seen in a 90-day study in dogs receiving doses of up to 5000 mg/kg, equal to 198 mg/kg bw per day. In a 1-year study in dogs fed diets containing methoxyfenozide at concentrations of 0–30 000 mg/kg (0, 60, 300, 3000, 30 000 mg/kg), there was evidence of haemolysis, methaemoglobinaemia, increased concentrations of bilirubin in blood and urine and increases in numbers of platelets. The presence of increased quantities of iron-positive pigment in the liver and spleen is consistent with phagocytosis of damaged erythrocytes. The NOAEL was 300 mg/kg, equal to 9.8 mg/kg bw per day, with a LOAEL of 3000 mg/kg, equal to 106 mg/kg bw per day. Extensive reversibility of haematological effects was demonstrated in dogs examined 4 weeks after the end of a 4-week exposure to 30 000 mg/kg, equal to 1036 mg/kg bw per day, of methoxyfenozide in the diet. This is consistent with the increases in reticulocytes and bone marrow hyperplasia observed in other studies and indicated that the effects on erythrocytes are not attributable to a direct effect on stem cells.

The chronic toxicity and carcinogenicity of methoxyfenozide was investigated in mice and rats given diets containing methoxyfenozide at concentrations equating to >1000 mg/kg bw per day in the groups receiving the highest dose. There was no treatment-related increase in the incidence of any tumour type. There were no significant treatment related non-neoplastic effects. The NOAEL for carcinogenicity and non-neoplastic effects in mice was 7000 mg/kg, equal to 1020 mg/kg per day, the highest dose tested. The Meeting concluded that methoxyfenozide is not carcinogenic in mice.

In the study in rats, poor survival (<50% at week 90) in all groups resulted in the study being terminated at 99 weeks. This reduction in the duration of exposure reduced the power of the study, but the study was considered to be adequate for the assessment of car-

cinogenic potential in rats. Non-neoplastic findings were consistent with those of the short-term studies. Changes in erythrocyte parameters, increases in numbers of platelets, serum γ -glutamyl transferase activity, liver weight, hepatocyte hypertrophy, glomerular nephropathy, thyroid follicular hyperplasia and erosion of the glandular stomach were seen at doses of 8000mg/kg, equal to 411mg/kgbw per day, and above. The NOAEL for non-neoplastic effects was 200mg/kg, equal to 10mg/kgbw per day. There was no treatment-related increase in the incidence of any tumour type. The NOAEL for neoplastic effects was 20000mg/kg, equal to 1045mg/kgbw per day. The Meeting concluded that methoxyfenozide was not carcinogenic in rats.

Methoxyfenozide (purity, 99%) has been investigated in an adequate range of studies of genotoxicity in vitro and in vivo and was found to give negative results. The Meeting noted that the purity of the material tested was greater than that of the proposed technical specification, but that the impurity profile (qualitative and quantitative) of the technical material (97%) did not give rise to any significant concerns regarding genotoxicity. The Meeting concluded that methoxyfenozide (technical material) was unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity observed in studies in rats and mice, the Meeting concluded that methoxyfenozide is not likely to pose a carcinogenic risk to humans.

A two-generation study of reproductive toxicity in rats treated with methoxyfenozide showed that there were no adverse effects on estrus cycling, sperm parameters, mating performance, litter size, pup body weight, pup viability or pup gross pathology at doses of up to 20000mg/kg, equal to 1474mg/kgbw per day. A significant increase in absolute and relative liver weights and altered liver histopathology were seen in parental animals at 20000mg/kg. The only compound-related effect observed in pups was a slight delay in attainment of vaginal patency, noted in both generations at a dose of 20000mg/kg, the values being outside the range for historical data for the test facility. The developmental delay in attainment of vaginal patency did not have any impact on reproduction at the second mating. There was no evidence that developing pups or second-generation parents were especially sensitive to methoxyfenozide. The NOAEL for reproductive effects was 20000mg/kg, equal to 1474mg/kgbw per day, the highest dose tested. The NOAEL for pup development was 2000mg/kg, equal to 143mg/kgbw per day, on the basis of delayed vaginal patency at 20000mg/kg. The NOAEL for parental toxicity was 2000mg/kg on the basis of increased liver weights and histopathological changes at 20000mg/kg.

The developmental toxicity of methoxyfenozide was investigated in rats and rabbits. Some marginal increases in fetal alterations were noted, but these were within the range of values for historical controls and were not of toxicological concern. There was no evidence of maternal toxicity at the limit dose of 1000mg/kgbw per day used in both studies. The overall NOAEL was 1000mg/kgbw per day. The Meeting concluded that methoxyfenozide is not teratogenic.

Methoxyfenozide was tested in studies of neurotoxicity, although there were no signs of neurotoxicity induced by methoxyfenozide in routine studies of toxicity. No evidence of neurotoxicity or neuropathy was seen at 2000mg/kgbw, the highest dose tested in a study of acute neurotoxicity in rats, or at 20000mg/kg, equal to 1318mg/kgbw per day, the highest dose tested in a 90-day study of neurotoxicity in rats receiving repeated doses of methoxyfenozide. No haematological investigations were performed in these studies.

The animal, soil and plant metabolite, *N*-2,3-hydroxybenzoyl-*N'*-3,5-dimethylbenzoyl-*N'*-*tert*-butylhydrazine (M14) has low acute oral toxicity in mice (LD_{50} , >5000 mg/kg bw), and was not mutagenic in an Ames test. It is expected that the metabolites of methoxyfenozide identified in rats will be of no greater toxicity than the parent compound.

Methoxyfenozide is a new compound and there has been only limited exposure of humans to this pesticide. No adverse findings have been identified during routine medical monitoring of workers and operators.

The Meeting concluded that the existing database on methoxyfenozide was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for methoxyfenozide of 0–0.1 mg/kg bw based on the NOAELs of 200 mg/kg, equal to 10 mg/kg bw per day, for effects on erythrocytes plus liver and thyroid hypertrophy in the long-term study in rats, and 300 mg/kg, equal to 9.8 mg/kg bw per day, for haematological effects in the 1-year study in dogs, and a 100-fold safety factor.

The Meeting concluded that the toxicological profile of methoxyfenozide required the derivation of an acute reference dose (RfD). The most appropriate end-point was considered to be haematotoxicity, for which the dog is the most sensitive species. In view of the fact that a 1-day study in dogs was available for the closely related compound, tebufenozide, which has a similar toxicity profile on repeated dosing, the Meeting decided to use this study to establish the acute RfD for methoxyfenozide. An acute RfD of 0.9 mg/kg bw was established, on the basis of the lack of haematological effects at 4300 mg/kg, equal to 89.4 mg/kg bw, and using a safety factor of 100. The Meeting noted that this value was likely to be conservative since tebufenozide was more potent than methoxyfenozide in producing effects on erythrocytes.

Levels relevant to risk assessment

Species	Study ^a	Effect	NOAEL	LOAEL
Mouse	78-week study of chronic toxicity and carcinogenicity	Toxicity and carcinogenicity	7000 mg/kg ^b , equal to 1020 mg/kg bw per day	—
Rat	2-year study of chronic toxicity and carcinogenicity	Toxicity	200 mg/kg, equal to 10 mg/kg bw per day	8000 mg/kg, equal to 411 mg/kg bw per day
		Carcinogenicity	20 000 mg/kg ^b , equal to 1945 mg/kg bw per day	—
	Two-generation study of reproductive toxicity	Parental and offspring toxicity	2000 mg/kg, equal to 143 mg/kg bw per day	20 000 mg/kg, equal to 1474 mg/kg bw per day
Dog	1-year study of toxicity	Toxicity	300 mg/kg, equal to 9.8 mg/kg bw per day	3000 mg/kg, equal to 106 mg/kg bw per day
	Single dose study with tebufenozide	Toxicity	4300 mg/kg ^b , equal to 89.4 mg/kg bw	—

^a All studies investigated dietary administration of methoxyfenozide

^b Highest dose tested

Estimate of acceptable daily intake for humans

0–0.1 mg/kg bw

Estimate of acute reference dose

0.9 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

Observations in humans

Summary of critical end-points for methoxyfenozide

<i>Absorption, distribution, excretion and metabolism in mammals</i>	
Rate and extent of absorption:	About 60–70% within 72 h in the rat (including biliary excretion of 40–65%) at a dose of 10 mg/kg bw
Distribution:	Widely distributed; highest absorbed concentrations after 15 min–2 h in the liver
Potential for accumulation	Low potential: <0.1% in liver after 5 days
Rate and extent of excretion:	Rapid: 60–80% in 24 h, mainly in the faeces
Metabolism in animals	Extensive (no parent found in urine or bile) Little cleavage of parent
Toxicologically significant compounds	Methoxyfenozide
<i>Acute toxicity</i>	
Rat, LD ₅₀ , oral	>5000 mg/kg bw
Rat, LD ₅₀ , dermal	>5000 mg/kg bw
Rat, LC ₅₀ , inhalation	>4.3 mg/l (4-h exposure, nose only, maximum achievable concentration)
Skin sensitization	Not sensitizing (Magnusson & Kligman test)
<i>Short-term studies of toxicity</i>	
Target/critical effect	Liver (hypertrophy), erythrocytes (methaemoglobin and haemolysis)
Lowest relevant oral NOAEL	10 mg/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data
<i>Genotoxicity</i>	Not genotoxic
<i>Long-term toxicity and carcinogenicity</i>	
Target/critical effect	Erythrocytes (reduced parameters), liver (hypertrophy), thyroid (hypertrophy)
Lowest relevant NOAEL	10 mg/kg bw per day (80–90 week study in rats)
Carcinogenicity	Not carcinogenic
<i>Reproductive toxicity</i>	
Reproduction target/critical effect	Delayed attainment of vaginal patency Parental hepatotoxicity
Lowest relevant NOAEL for reproductive toxicity	143 mg/kg bw per day
Developmental target/critical effect	No embryotoxicity or fetotoxicity Not teratogenic
Lowest relevant NOAEL for developmental toxicity	1000 mg/kg bw per day (highest dose tested in rats and rabbits)
<i>Neurotoxicity/delayed neurotoxicity</i>	
Acute neurotoxicity	NOAEL: >2000 mg/kg bw; no neuropathy (rat)
90-day study of neurotoxicity	NOAEL: 1318 mg/kg bw per day (highest dose tested); no neuropathy (rat)
<i>Other toxicological studies</i>	
Tebufenozide single dose study in dogs	No effects at 89.4 mg/kg bw (highest dose tested)
Metabolite: N-2,3-hydroxybenzoyl-N'-3,5-dimethylbenzoyl-N'-tert-butylhydrazine (M14)	Acute LD ₅₀ >5000 mg/kg bw in mice treated orally; not mutagenic in an Ames test
<i>Medical data</i>	No adverse effects reported but data limited (new compound)

Summary	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw	Rat, long-term study; and dog, 1-year study Dog, single dose of tebufenozide	100
Acute RfD	0.9 mg/kg bw		100

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PARAQUAT

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Explanation

Paraquat is a bipyridilium herbicide that was evaluated by the JMPR in 1970, 1972, 1976, 1985 and 1986 (Annex 1, references 14, 18, 26, 47), in order to establish an acceptable daily intake (ADI). A toxicological monograph was published after the 1970 JMPR and addenda to the monograph were published after the 1972, 1976 and 1982 Meetings. A toxicological monograph was published after the 1986 JMPR. At the JMPR in 1970, an ADI of 0–0.001 mg/kg bw, as paraquat dichloride, was established. The 1972 JMPR assigned an ADI of 0–0.002 mg/kg bw, while the 1982 JMPR reduced the ADI to 0–0.001 mg/kg bw. The 1986 JMPR established an ADI of 0–0.004 mg/kg bw as paraquat ion (equal to 0–0.006 mg/kg bw as the dichloride).

Paraquat was re-evaluated by the present Meeting within the periodic review programme of the Codex Committee on Pesticide Residues. A considerable amount of data has been generated since 1986 and was submitted for evaluation; these data include studies on the absorption, distribution, metabolism and excretion of paraquat and numerous studies of toxicity (acute, reproductive and developmental). Furthermore, a substantial number of papers in the open literature on, inter alia, the genotoxicity and neurotoxicity of paraquat have been reviewed. In all studies relevant to risk assessment, doses and intakes are expressed as paraquat ion.¹

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

In a study of the absorption, distribution and excretion of paraquat, a single oral dose of ¹⁴C-labelled paraquat ion at 1 mg/kgbw was administered to five male and five female Alpk:ApfSD rats by gavage. Paraquat dichloride was used as the test material; the purity of the ¹⁴C-labelled material was 100%, while that of the unlabelled material was >96%. The specific activity of the radiolabelled material was 4.0996 GBq/mmol and that of the dosing solution was 4.12 MBq/g. Urine was collected 6 h after dosing and urine and faeces were collected separately at 12, 24, 36, 48 and 72 h after dosing. The animals were killed after 3 days and selected organs and tissues were removed. The amount of radioactivity remaining in the blood, selected tissues and the carcasses was estimated. Excretion of the radiolabel was rapid: in the first 24 h, in males 17.9% of the dose was excreted in the urine and 63.1% in the faeces. Equivalent figures for females were 11.6% and 74.1%. More than 90% of the radiolabel was eliminated in 72 h in both sexes. More radiolabel was excreted in the faeces of females than males. Only low concentrations of radiolabel were retained in the residual carcasses (0.64% and 0.54% of the administered dose in male and females respectively), the highest concentrations (0.01–0.02%) being found in the liver, lungs and kidneys (Lythgoe & Howard, 1995a).

In a second study of the absorption, distribution and excretion of paraquat, daily oral doses of paraquat (1 mg of paraquat ion/kgbw) were administered by gavage to eight male and eight female Alpk:ApfSD rats for 14 days. Paraquat dichloride was used as the test material; the purity of the ¹⁴C-labelled material was 100%, while that of the unlabelled material was >96%. A single oral dose of ¹⁴C-labelled paraquat ion at 1 mg/kgbw was subsequently administered by gavage. The specific activity of the radiolabelled material was 4.0996 GBq/mmol and that of the dosing solution was 4.12 MBq/g. Urine was collected 6 h after dosing and urine and faeces were collected separately at 12, 24, 36, 48 and 72 h after dosing. The animals were killed after 3 days and selected organs and tissues were removed. The amount of radioactivity remaining in the blood, selected tissues and the carcasses was estimated. Excretion of the radiolabel was rapid: in the first 24 h, in males, 18.8% of the dose was excreted in the urine and 68.3 % in the faeces. Equivalent figures for females were 10.3% and 70.7%. Of the radiolabel, 92.5% was eliminated within 72 h in the male rats and

¹ The relative molecular mass of paraquat is 186.3; the relative molecular mass of paraquat dichloride is 257.2; therefore 1 g of paraquat dichloride = 0.724 g of paraquat ion.

93.9% in female rats. Tissue concentrations of radiolabel were generally lower in the females than in males. Only low concentrations of radiolabel were retained in the residual carcass (0.70% and 0.55% of the administered dose in males and females, respectively), the highest concentrations being found in the lungs, livers and kidneys (Lythgoe & Howard, 1995b).

In a third study of the absorption, distribution and excretion of paraquat, a single dose of ^{14}C -labelled paraquat (50 mg of paraquat ion/kg bw) was administered by gavage to five male and five female Alpk:ApfSD rats. The specific activity of the dosing solution was 79.83 kBq/g. Urine was collected 6 h after dosing and urine and faeces were collected separately at 12, 24, 36, 48 and 72 h after dosing. The animals were killed after 3 days and selected organs and tissues were removed. The amount of radioactivity remaining in the blood, selected tissues and the carcasses was estimated. Excretion of the radiolabel was rapid: in the first 24 h, in males, 9.2% of the dose was excreted in the urine and 54.5 % in the faeces. Equivalent figures for females were 11.6% and 49.6%. Of the label, 92.7% was eliminated in 72 h in the male rats and 91.7% in female rats. The highest concentrations of radioactivity were retained in the lungs and residual carcass (Lythgoe & Howard, 1995c).

Daniel & Cage (1966) investigated the absorption and excretion of paraquat (and diquat) in albino Wistar rats given ^{14}C -labelled paraquat dichloride (0.94 mCi/mmol) as single oral doses at 4 or 6 mg/kg bw, or paraquat dimethosulfate as oral doses at 2.5–24 mg/kg bw, or subcutaneously at a dose of 21 or 23 mg/kg bw. Paraquat was poorly absorbed from the gut. After administration by either route, most of the radiolabel was found in the excreta within 2 days. After oral administration of paraquat, no radiolabel was detected in the bile (Daniel & Cage, 1966).

Dey et al. (1990) studied the pharmacokinetics of ^{14}C -labelled paraquat (111 mCi/mmol) administered to male Sprague-Dawley rats as a single subcutaneous injection at a dose of 72 $\mu\text{mol/kg bw}$. This dose was considered to be one that would produce lung damage but avoid kidney damage. Blood was sampled through indwelling cannulae, and urine and faeces were collected at 2, 4, 6, 8, 12, and 24 h and then daily for 7 days. Non-cannulated rats treated in the same way were exsanguinated at intervals from 10 min to 7 days after dosing; tissue concentrations of ^{14}C were measured in selected organs. The right lungs and kidneys were processed for histopathological examination. Histopathological examination showed changes characteristic of paraquat-induced lung pathology, without renal damage. Paraquat was rapidly absorbed, with peak blood concentrations of 58 $\mu\text{mol/l}$ after 20 min. The pharmacokinetics were best characterized as a two-compartment open model, the mean half-life ($t_{1/2}$) being approximately 40 h. Highest tissue concentrations observed were in the kidney (358 nmol/g of tissue) and lung (64 nmol/g tissue), both at 40 min after administration of paraquat (Dey et al., 1990).

The distribution of paraquat in the brain was examined in male adult Wistar-derived Alderley Park rats after subcutaneous administration of paraquat (containing ^{14}C -labelled paraquat with a specific activity 2 mCi/mmol) at a dose of 20 mg of ion/kg bw. The aim of this study was to determine whether paraquat crosses the blood–brain barrier. After administration, the concentration of radiolabel in the brain reached a maximum (0.05% of administered dose) within the first hour and then rapidly disappeared. Twenty-four hours after administration, however, a residual amount of paraquat still remained in the brain (1.6 nmol/g wet weight) and could not be removed by intracardiac perfusion. Most of the paraquat was associated with five structures, two of which (the pineal gland and linings of the cerebral ventricles) lie outside the blood–brain barrier. The remaining three brain areas

(the anterior portion of the olfactory bulb, hypothalamus and area postrema) do not have a blood–brain barrier. Overall, the distribution of ^{14}C -labelled paraquat in the brain 24 h after systemic administration was highly correlated to the blood volume. The authors concluded that paraquat remaining in the brain 24 h after systemic administration was associated with elements of the cerebral circulatory system, such as the endothelial cells that make up the capillary network, and that there was limited entry of paraquat into brain regions without a blood–brain barrier (Naylor et al., 1995).

The extent to which paraquat entered the brain was compared in groups of neonatal (aged 10 days), adult (aged 3 months) and elderly (aged 18 months) Wistar-derived Alpk: ApfSD rats. Both male and female neonatal rats were studied, while the adult and elderly rats were males. Groups of six to eight rats were given a single dose of [^{14}C]paraquat (103 mCi/mmol) at 20 mg/kg, administered subcutaneously, and killed 30 min or 24 h after injection; blood was taken by cardiac puncture and brains were removed. Groups of four neonatal, adult or elderly rats were similarly injected and killed 24 or 48 h after dosing; the brains of these animals were subjected to histopathological examination. At all ages, plasma concentrations of paraquat were much higher at 30 min than at 24 h. At 30 min, the concentration of paraquat in the brain was highest in the elderly rats. While at 24 h the concentration in the brains of the adult and elderly rats had decreased, it remained high in the brains of the neonatal rats. Autoradiography showed similar distributions of paraquat in the various brain regions, paraquat being found in areas outside the blood–brain barrier or where the barrier is incomplete, e.g. the dorsal hypothalamus, area postrema and anterior olfactory bulb. There was no evidence for paraquat-induced cell damage in the neonatal brain, although there was increased paraquat entry into the brain in neonates than in older rats (Widdowson et al., 1996a).

In a study of the entry of paraquat into the brain, five male Wistar-derived Alpk: ApfSD rats were given paraquat (labelled with [^{14}C]paraquat; specific activity, 20 $\mu\text{Ci/ml}$) at a dose of 5 mg of ion/kg bw per day) daily for 14 days by oral administration, and another five rats received a single oral dose of paraquat (labelled with [^{14}C]paraquat; specific activity 106 $\mu\text{Ci/ml}$) at a dose of 5 mg ion/kg bw. The rats were killed 24 h after the last of the 14 doses or after the single dose. Concentrations of paraquat in the brain were 10 times higher in rats receiving multiple doses than in those receiving single doses. The same paper described a study of neuropathology, which included behavioural tests (see below) (Widdowson et al., 1996b).

In a study that used a brain microanalysis technique with detection by high-performance liquid chromatography–ultraviolet (HPLC–UV), paraquat, administered subcutaneously at a dose of 5, 10 or 20 mg/kg bw, was found to appear in the dialysate of the striatum in male Wistar rats. It was also found that paraquat did not allow 1,2,3,6-tetrahydropyridinium ion to penetrate the blood–brain barrier. Intraperitoneal injection of L-valine (200 mg/kg) 30 min before administration of paraquat at a dose of 20 mg/kg bw reduced the striatal extracellular concentrations of paraquat. The authors hypothesized that paraquat is taken up into the brain via the neutral amino acid transporter (Shimizu et al., 2001; see also McCormack & Di Monte, 2002).

In a study in anaesthetized male Wistar rats, the excretion of paraquat was found to be greater than the glomerular filtration rate, and to be concentration-dependent and saturable, implying that paraquat is secreted by a process involving active transport (Chan et al., 1997).

Groups of albino Wistar rats were given diets containing paraquat at a concentration of 50, 120 and 250 mg/kg (as paraquat ion) for 8 weeks. Groups comprised 30 animals at the two lower dietary concentrations and 40 animals at the highest concentration. After 2, 4 and 8 weeks, 10 rats per group were killed and selected organs were analysed for paraquat. At 50 mg/kg, paraquat was not detected in the kidneys, liver, brain or lung at any time, but was present in the gastrointestinal tract and, at low concentrations, in muscle. At 120 mg/kg, paraquat was detected in kidneys, lung and gastrointestinal tract. At 250 mg/kg, paraquat was detected in kidneys, lung and gastrointestinal tract (Litchfield et al., 1973).

Mice

The tissue distribution of paraquat was studied using whole body autoradiography in mice treated by intravenous injection. Mice received ^{14}C -labelled paraquat at a dose of 20 mg of paraquat ion/kg. Two mice were killed at each time-point after the paraquat injection (10 min, 1, 5, 24 and 72 h). Paraquat was found to be concentrated in the liver and cartilage, and was not detected in the central nervous system. Paraquat was also present in the lungs, notably so after 24 h. At 72 h, radioabel was only present in the stomach and intestinal contents (Litchfield et al., 1973).

Hens

Three Warren hens were given gelatin capsules containing ^{14}C -ring-labelled paraquat (purity, 99.7%; specific activity, 1.216×10^5 dpm/ μg) at a daily dose of 4.52 mg of paraquat ion (0.247 mCi) for 10 days. One hen was used as the control. The hens were killed 4 h after the last dose. The highest concentration of radiolabel was found in the kidneys, while rather less was found in the gizzard and liver. Very little was found in fat. Paraquat was found at a concentration of 0.052 $\mu\text{g/g}$ in eggs, mostly in the yolk (Hendley et al., 1976b).

Dogs

Greyhound dogs were given ^{14}C -labelled paraquat at a dose of 30–50 $\mu\text{g/kgbw}$. The authors of this study considered that the kinetics could be described by a three-compartment open linear system (Bennett et al., 1976).

The elimination of paraquat was studied in the female greyhound dog. After intravenous injection of low doses (30–50 $\mu\text{g/kg}$) of ^{14}C -labelled paraquat, radiolabel was found to be rapidly excreted in the urine, the clearance being greater than the glomerular filtration rate, suggesting a process of active secretion. Secretion could be inhibited by *N*'-nicotinamide. Large doses of paraquat (20 mg/kgbw) produced renal failure, as evidenced by a decrease in both renal creatinine and paraquat clearance. The elimination of paraquat could be described by a three-compartment open model (Hawksworth et al., 1981).

Goats

An English white nanny goat was dosed with ^{14}C -ring-labelled paraquat (purity, 99.7%; specific activity, 2.28×10^4 dpm/ μg) in the diet at a dose equivalent to 100 μg of paraquat ion/g of diet. This was done by adding 206.6 mg of radiolabelled paraquat (as ion) to the diet at the morning and afternoon feeding, daily for 7 days. Another nanny goat was used as the control. The goats were killed 4 h after the last feeding with radiolabelled paraquat. Radioactivity was measured in the urine, faeces, stomach, milk, and in selected tissues. At sacrifice, 2.4% and 50.5% of the administered material had been excreted in the urine and faeces respectively. The stomach contents included 33.2% of the administered

dose. The highest concentration of radiolabel seen in the milk was $0.009\mu\text{g/g}$ (on the morning of day 7). The highest tissue concentrations were found in the kidney and liver (Hendley et al., 1976a).

Pigs

A pig (Large White \times Welsh boar) was given 100 mg of ^{14}C -methyl-labelled paraquat (purity, 99.3%; specific activity, $4.88 \times 10^4 \text{ dpm}/\mu\text{g}$ of paraquat ion) on 7 consecutive days; this was calculated to be equivalent to about $50\mu\text{g}$ of paraquat ion/g of diet. A second boar acted as the control. The daily dose was spotted onto the commercial pig diet. The pig was killed 2 h after the final dose. The highest concentrations of paraquat were present in the kidney and liver (Leahey et al., 1976).

In a second study in pigs, ^{14}C -methyl-labelled paraquat dichloride (purity, 99.45%; specific activity, $4.72 \text{ dpm}/\mu\text{g}$) at a daily dose of approximately 100 mg of paraquat ion was administered twice daily for 7 days to a Large White \times Welsh boar. The dose contained about 2 mCi of radiolabel and the content of paraquat was calculated to be equivalent to about $50\mu\text{g}$ paraquat ion/g of diet. The daily dose was spotted onto commercial porcine diet pellets. A second boar acted as the control. The highest concentrations of radiolabel were found in the kidney, with somewhat less being found in the liver and lung (Spinks et al., 1976).

Monkeys

Purser & Rose (1979) studied the renal handling of paraquat administered orally at a dose of 85 mg of paraquat ion/kg bw (containing $500\mu\text{Ci}$ of ^{14}C -labelled paraquat) to three male cynomolgus monkeys (*Macaca fascicularis*). In two monkeys, peak plasma concentrations were observed at 2 h in two monkeys and at 10 h in the third monkey. The renal clearance of paraquat was high during the first 10 h, but fell markedly as renal failure set in at 14 h. The clearance of paraquat was always well in excess of the clearance of creatinine, suggesting an active secretory process.

Studies in more than one species

The disposition of orally-administered ^{14}C -labelled paraquat dichloride was studied in male Sprague-Dawley rats, male and female guinea-pigs, and monkeys (*Macaca fascicularis*). The doses used were: rats, 126 mg/kg bw ($0.175\mu\text{Ci}/\text{mg}$); guinea-pigs, 22 mg/kg bw ($1.25\mu\text{Ci}/\text{mg}$); and monkeys, 50 mg/kg bw ($0.4\mu\text{Ci}/\text{mg}$). In the case of the rats and guinea-pigs, the doses used were LD_{50} s at 7 days. A total of 61 rats, 21 guinea-pigs and three monkeys were used. For the rats and guinea-pigs, urine and faeces were collected and groups were sacrificed at various times up to 21 days after the administration of paraquat. Selected organs were collected at sacrifice. For the monkeys, blood samples were taken at 30 min, 1, 2, 4, 8, 16 and 32 h after administration of paraquat and daily thereafter. In the rats, deaths were seen mainly after 5 days. A large portion of the paraquat was not absorbed from the gastrointestinal tract. Peak serum concentrations of radiolabel were seen at 30–60 min, while concentrations of radiolabel were higher in liver, kidneys and lungs than in serum. Similar results were found in the guinea-pigs. In the monkeys, one of which died on day 8, serum concentrations of radiolabel decreased rapidly after the first time-point (Murray & Gibson, 1974).

There is evidence that paraquat is taken up into the lungs by a process of active uptake, the normal substrate being endogenous diamines, e.g. putrescine and polyamines such as spermine and spermidine (see review by Smith, 1985). Diquat is not a substrate for this system and this fact accounts for the different organ-specific toxicity of these two bipyridilium herbicides (this is discussed further below).

1.2 Biotransformation

Rats

In the Daniel & Cage (1966) study in albino Wistar rats treated with ^{14}C -labelled paraquat dichloride, discussed above, some evidence of metabolism was found. Of the dose of paraquat administered orally, 30% of the radiolabel was present in the gut as metabolic products. Furthermore, a small amount of metabolite was present in the urine after oral but not subcutaneous administration, suggesting that metabolites were absorbed from the gut. Studies in vitro, using faecal homogenates, suggested that microbiological metabolism was responsible for this. In the study of Murray & Gibson (1974) in male Sprague-Dawley rats, male and female (mixed) guinea-pigs and cynomolgus monkeys (*Macaca fascicularis*), metabolites were not observed.

Urine and faeces samples from the studies in rats, described above (Lythgoe & Howard, 1995a, b, c), were pooled separately for the females and males of each study for the whole 72h of that study. After extraction, samples were analysed by thin-layer chromatography. In all cases, paraquat accounted for the vast majority of the radiolabel in the urine (95.0% of urinary label in the males receiving the lower dose and 93.6% females receiving the lower dose). Three minor metabolites were found in urine; these were not further identified. Faecal material showed that the vast majority of the radioactivity in all cases was unchanged paraquat. It was therefore concluded that paraquat was largely unmetabolized (Macpherson, 1995).

Hens

In the study in hens, residues in tissues were generally unchanged paraquat. A small amount of a metabolite, 1-methyl-(4'-pyridyl), was found in the livers and kidneys (Hendley et al., 1976b).

Goats

In the study in goats, residues in tissues were generally unchanged paraquat. In the liver, small amounts of 4-(1,2-dihydro-1-methyl-2-oxo-4-pyridyl)-1-methyl pyridinium ion and 1-methyl-4-(4'-pyridyl) pyridinium ion were found. The latter compound was also found in peritoneal fat (Hendley et al., 1976a).

Pigs

In the study by Leahey et al. (1976), all the radiolabel in the tissues, except the liver, was found to be in the form of paraquat. In the liver, 7% of the radiolabel was accounted for by 1-methyl-4-(4'-pyridyl) pyridinium ion. In the study by Spinks et al. (1976), 4% of the radiolabel was accounted for by 1-methyl-4-(4'-pyridyl) pyridinium and 70% by unchanged paraquat.

2 Toxicological studies

2.1 Acute toxicity

The results of studies on the acute toxicity of paraquat administered by a variety of routes are summarized in Table 1. Clinical signs seen after administration of paraquat by the oral, subcutaneous or intraperitoneal routes included decreased activity, dehydration and breathing irregularity. In animals that died after administration of paraquat by these routes, mottled areas of lung were seen. Scabbing of skin was seen after administration by the dermal route, but no systemic signs of poisoning were present. After rats had inhaled paraquat, clinical signs and appearances post mortem were similar to those seen after oral, subcutaneous or intraperitoneal administration.

(a) Dermal irritation

The dermal irritation potential of paraquat dichloride technical concentrate (paraquat ion, 33% w/w) was assessed in young adult female New Zealand white albino rabbits. Undiluted test material was applied to the depilated left flank of the rabbits, which was then covered by gauze and impermeable rubber. These were left in place for 4 h. After removal of the dressing and cleansing of the application site, the Draize scale was used to assess erythema and oedema, 30–60 min and 1, 2, 3, 4, 7, 14, 17, 20, 21 and 23 days after exposure. Slight erythema was observed, which regressed by 3 days and 4 days in two animals, but still remained after 23 days in the third animal. Very slight transient oedema was seen in one animal (at the 30/60 min observation time), very slight oedema was seen in the second, this still being present at 4 days but not at 7 days, while there was no oedema in the third rabbit (Duerden, 1994c).

(b) Ocular irritation

The potential for paraquat dichloride technical concentrate (paraquat ion, 33% w/w) to produce irritation of the eye was assessed in young adult female New Zealand white albino rabbits. Test material (0.1 ml) was applied to the left eye of each rabbit. Rabbits were dosed sequentially; and mild systemic toxicity was noted in the third rabbit to be dosed. Accordingly, this rabbit was killed. The fourth rabbit was collared to prevent oral ingestion of the test material. The eyes of rabbits 1, 2 and 4 were then examined and the degree of irritation was assessed using the Draize scale from 1 h to up to 28 days after instillation. Initial pain was graded as slight or was absent. Slight or mild corneal opacity was seen in all three animals, this effect resolving within 17 days. Redness and chemosis of the conjunctiva was seen in all animals and resolved by 28 days and 14 days after exposure. No effect was seen on the iris, while erythema of the eyelids and mucoid discharge was observed. Paraquat was considered to be a moderate ocular irritant (Bugg & Duerden, 1994).

In a study of ocular toxicity, paraquat was administered at a concentration of 6.25, 12.5, 25, 50 and 100% of a solution containing 242 mg of paraquat ion/ml. A total of 15 male New Zealand white rabbits were used, nine rabbits receiving different doses in each eye and six rabbits receiving the same dose in both eyes. Control eyes received normal saline. In all cases, 0.2 ml of solution was pipetted into the lower conjunctival sac, and the eyes were examined at 12 h and then daily for 20 days, ocular lesions being scored on the Draize scale. At 6.25 and 12.5%, severe conjunctival reactions were seen, with occasional slight corneal damage at 12.5%. At higher concentrations (25 and 50%), the iris was congested and swollen and there was corneal opacification; a pannus reaction was also seen. Animals to which the 100% solution was administered died within 6 days. The time of

Table 1. Acute toxicity of paraquat

Species	Strain	Sex	Route	LD ₅₀ (mg/kg bw) (95% confidence interval)	Reference
Mouse	ICR	M	Per os	360 (324–400) ^a	Shirasu & Takahashi (1977)
	ICR	F	Per os	290 (254–331) ^a	Shirasu & Takahashi (1977)
	ICR	M	Subcutaneous	41.0 (36.9–45.5) ^a	Shirasu & Takahashi (1977)
	ICR	F	Subcutaneous	36.8 (32.9–41.2) ^a	Shirasu & Takahashi (1977)
	ICR	M	Intraperitoneal	40.6 (35.6–46.3) ^a	Shirasu & Takahashi (1977)
	ICR	F	Intraperitoneal	39.2 (35.6–43.1) ^a	Shirasu & Takahashi (1977)
	Swiss-Webster	M	Intraperitoneal	39 (32.5–46.8)	Sinow & Wei (1973)
	Swiss-Webster	F	Intraperitoneal	30 (26.3–34.2)	Bus et al. (1976a)
Rat	NS	F	Per os	112 (104–122) ^b	Clark et al. (1966)
	NS	F	Per os	150 (139–162) ^b	Clark et al. (1966)
	Alpk: APfSD	M	Per os	344 (246–457) ^c	Duerden (1994a)
	Alpk: APfSD	F	Per os	283 (182–469) ^c	Duerden (1994a)
	Sprague-Dawley	M	Per os	223 (199–259) ^a	Shirasu & Takahashi (1977)
	Sprague-Dawley	F	Per os	258 (228–292) ^a	Shirasu & Takahashi (1977)
	Sherman	M	Per os	100 ^d	Kimbrough & Gaines (1970)
	Sherman	F	Per os	110 ^d	Kimbrough & Gaines (1970)
	NS	F	Per os	150 (110–173)	Mehani (1972)
	Sprague-Dawley	M	Per os	126	Murray & Gibson (1972)
	Sprague-Dawley	M	Subcutaneous	26.8 (23.9–30.0) ^a	Shirasu & Takahashi (1977)
	Sprague-Dawley	F	Subcutaneous	32.0 (28.1–36.5) ^a	Shirasu & Takahashi (1977)
	NS	F	Intraperitoneal	19 (16–21) ^b	Clark et al. (1966)
	Sprague-Dawley	M	Intraperitoneal	24.8 (21.8–28.3) ^a	Shirasu & Takahashi (1977)
	Sprague-Dawley	F	Intraperitoneal	26.8 (23.7–30.6) ^a	Shirasu & Takahashi (1977)
	NS	F	Intraperitoneal	16 (10–26)	Mehani (1972)
Rat	Sherman	M	Dermal	80 ^d	Kimbrough & Gaines (1970)
	Sherman	F	Dermal	90 ^d	Kimbrough & Gaines (1970)
	Alpk: APfSD	M	Dermal	>2000 ^e	Duerden (1994b)
	Alpk: APfSD	F	Dermal	>2000 ^e	Duerden (1994b)
	Alpk: APfSD	M	Inhalation	0.6–1.4 ^{ef}	McLean et al. (1985)
	Alpk: APfSD	F	Inhalation	0.6–1.4 ^{ef}	McLean et al. (1985)
Rabbit	NS	M	Per os	50 (45–58)	Mehani (1972)
	NS	M	Intraperitoneal	25 (15–30)	Mehani (1972)
Guinea-pigs	NS	M	Per os	30 (22–41) ^b	Clark et al. (1966)
	Sprague-Dawley	M & F	Per os	22	Murray & Gibson (1972)
	NS	F	Intraperitoneal	3 ^b	Clark et al. (1966)
Hens	Rhode Island	F	Per os	262 (200–346) ^b	Clark et al. (1966)
Turkeys	White	F	Per os	Approx. 290	Smalley (1973)
	White	F	Intraperitoneal	Approx. 100	Smalley (1973)
	White	F	Intravenous	Approx. 20	Smalley (1973)
	White	F	Dermal	375	Smalley (1973)
Cats	NS	F	Per os	35 (27–46) ^b	Clark et al. (1966)
Dog	Beagles	M	Subcutaneous	1.8 (1.0–6.1)	Nagata et al. (1992)
	Beagles	F	Subcutaneous	3.5 (2.4–10.1)	Nagata et al. (1992)
Monkeys	Cynomolgus (<i>M. fascicularis</i>)	M & F	Per os	50	Murray & Gibson (1972)
	Cynomolgus (<i>M. fascicularis</i>)	M	Per os	70 ^b	Purser & Rose (1979)

NS, not stated; M, male; F, female

^a Paraquat dichloride; purity, >98%^b Dose quoted as paraquat ion^c Technical paraquat dichloride (33% w/w paraquat ion)^d as dimethylsulfate^e LC₅₀ (at 4 h) (mg of paraquat ion/m³)^f Material used was paraquat dichloride, 21.5% w/v, but results were expressed as paraquat ion; aerosol mass median aerodynamic diameter (MMAD), <0.3 µm; rats exposed by nose only

maximal effect was around 9 days and those who received the 25% and weaker solutions showed recovery thereafter (Sinow & Wei, 1973).

(c) *Dermal sensitization*

A study of the sensitization potential of paraquat dichloride technical concentrate (paraquat ion, 33% w/w) was based on the maximization test of Magnusson & Kligman (1969). Female albino (Hsd/Poc:DH) guinea-pigs were used. The positive control was 2-mercaptobenzothiazole. A preliminary study was carried out to determine the concentrations of test material that gave an acceptable degree of irritancy and no signs of systemic toxicity. In the main study, 30 guinea-pigs were used (20 as test animals and 10 as controls). For induction, each animal received Freund complete adjuvant diluted 1:1 with deionized water, 0.03% w/v test material, and 0.03% w/v test material with Freund complete adjuvant 1:1 with deionized water, which were injected intradermally at three different sites in the previously depilated scapula region. One week later, the scapula region was again clipped and the test material (10% w/v) was applied topically over the injection sites. Animals serving as negative controls were treated in the same way except that the three inducing injections were Freund complete adjuvant 1:1 with deionized water, deionized water, and again Freund complete adjuvant 1:1 with deionized water. Animals serving as positive controls (20) were treated in the same way as the test animals except that the test substance administered was 2-mercaptobenzothiazole, and there were 10 negative controls for this group. For these guinea-pigs, the topical applications consisted of deionized water. Two weeks after the topical applications, both flanks of all animals were clipped free of hair and a preparation of 30% test material on an occlusive dressing was applied to one flank and a preparation of 10% test material to the other flank. These were left in place for 24 h. Erythematous reactions were recorded at 24 h and 48 h later. One animal in the test group died, but no erythema was found at either time in this group, nor in the negative control group. In contrast, erythema was seen in 19 of the positive controls, and it was concluded that paraquat had no sensitization potential (Duerden, 1994d).

2.2 *Short-term studies of toxicity*

(a) *Oral administration*

Mouse

In a 13-week dietary study, groups of 20 male and 20 female ICR-CRJ SPF mice were given paraquat dichloride (purity, 93.3%) at a dietary concentration of 0, 10, 30, 100 and 300 mg/kg, equal to 1.18, 3.65, 11.5 and 35.8 mg of paraquat dichloride/kg bw per day in males and 1.38, 3.91, 13.8 and 41.9 mg of paraquat dichloride/kg bw per day in females. These doses are equal to 0, 0.85, 2.64, 8.33 and 25.9 mg of paraquat ion/kg bw per day in males and 0, 1.00, 2.83, 9.99 and 30.3 mg of paraquat ion/kg bw per day in females. Mice were observed daily for mortality and daily clinical observations were undertaken. Animals found dead or that were killed in extremis were subjected to immediate autopsy. The mice were weighed weekly and food and water consumption were measured twice per week. On day 91, blood was collected from at least 10 mice from each group for haematological examination and for clinical chemistry. The mice were then examined post mortem. Autopsy was carried out on the remainder of the mice the next day, at which time urine was collected for urine analysis. At necropsy, selected organs were weighed and these and other organs were fixed and sections made for histopathological examination. Mortality was observed at 300 mg/kg, two females dying from pulmonary damage, one in week 2 and one in week 11. The decedents' lungs showed pulmonary oedema, small round cell infiltration with phago-

cytosis, and, in one animal, eosinophilic swelling of the epithelial cells of the alveoli. At 300 mg/kg in both sexes, there was reduced body-weight gain, almost from the inception of the study, however, these values were only significantly different from those of controls at a few time intervals. No intergroup difference in food intake was observed, but a slight reduction in food conversion efficiency was seen at 300 mg/kg in both sexes. No intergroup differences were seen in water intake. No test material-related intergroup differences were seen in haematological parameters (although a reduction in mean corpuscular volume at 300 mg/kg may have been test material-related in males) or in clinical chemistry findings. Terminal body weights were reduced in males at the highest dietary concentration, as were the absolute weights of the heart, liver and muscle. An increase in relative lung weight and a decrease in relative liver weight were also seen. In females at the highest dietary concentration, an increase in absolute pituitary, lung, kidney and spleen weight was observed, accompanied by a decrease in ovarian weight. Relative weights of organs also showed some intergroup differences, those of the pituitary, thyroids, lung, kidneys and spleen being increased while that of the ovaries was decreased. Eosinophilic hypertrophy of alveolar epithelial cells was observed in both sexes (17 out of 20 males, and 12 out of 18 surviving females). Pulmonary oedema and alveolar cell proliferation was also seen in a few males and in one female. Accordingly, the no-observed-adverse-effect level (NOAEL) for the study was 100 mg/kg (equal to 11.5 mg of paraquat dichloride/kg bw per day for males and 13.8 mg of paraquat dichloride/kg bw per day for females), on the basis of decreased body-weight gain and histopathological changes in the lungs at 300 mg/kg. These NOAELs are equal to 8.33 mg of paraquat ion/kg bw per day in males and 9.99 mg of paraquat ion/kg bw per day in females (Maita et al., 1980a).

Rat

In a 13-week study, groups of 20 male and 20 female Fischer CDF (F344) CRJ rats were given diets containing paraquat dichloride (purity, 93.8%) at a concentration of 0, 10, 30, 100 or 300 mg/kg (0, 7, 22, 72 and 217 mg/kg of paraquat ion, equal to 0, 0.49, 1.44, 4.74, 14.2 mg of paraquat ion/kg bw per day for males and 0, 0.52, 1.53, 5.14 and 15.27 mg of paraquat ion/kg bw per day for females). Another group received diet containing no test material and acted as controls. The rats were examined daily for adverse clinical signs, body weight was measured weekly and food and water intake were measured twice per week. Ninety-one days after the start of the study, at least 10 animals of each sex per group were chosen for blood sampling. The samples were used for haematology and clinical chemistry and, after sampling, the animals were examined post mortem. On day 92, urine analysis was carried out on the remaining rats, which were examined post mortem. At necropsy, selected organs were weighed and these and other organs were fixed in 10% formalin; they were then processed for histopathological examination. No rats died during the study and no abnormal clinical signs were seen. At the highest dietary concentration there was markedly reduced body-weight gain and decreased food and water intake in both sexes. Neither reduced body-weight gain nor reduced food intake was seen at lower dietary concentrations. No test material-related abnormalities were found on haematological examination, clinical chemistry or urine analysis. In the males, terminal body weights and absolute weights of brains, pituitaries, thyroids, livers, kidneys, spleens and muscles were decreased at the highest dietary concentration. Also in males, relative weights of brain, pituitary, lung, kidneys, adrenals, testes and muscle were increased. In females, terminal body weights were depressed at the highest dietary concentration, together with the absolute weight of the heart, lung and liver. Relative brain, lung, kidney, ovary and muscle weights were increased at the highest dietary concentration. These changes proba-

bly reflected the reduced food intake at the highest dietary concentration. On histopathological examination, alveolar epithelial hypertrophy was observed in males (6 out of 20) while in females, there was an increased prevalence of brown pigmentation of the spleen, both at the highest dietary concentration. The NOAEL was therefore 100mg/kg in both sexes, equal to 4.74mg of paraquat ion/kgbw per day for males and 5.14mg of paraquat ion/kgbw for females on the basis of reduced body-weight gain and reduced food and water intake at the highest dietary concentration, together with pathological changes in the lungs and spleen (Maita et al., 1980b).

Dog

In a 6-week study, groups of three male and three female beagle dogs received technical-grade paraquat (purity, 32.2%) at a dietary concentration of 35 or 90mg/kg as paraquat ion (equivalent to 0.875 and 2.25mg of paraquat ion/kgbw per day) for 6 weeks. An additional group of three males and three females received capsules containing paraquat at a dose of 0.75mg of paraquat ion/kgbw per day, also for 6 weeks. The controls from Sheppard (1981b) were used (see below) and the results were also compared with the group receiving paraquat at 20mg/kg in that study, as this is comparable to the dose of 0.75mg/kg bw per day in capsules. Animals were observed periodically during the working day, and by a veterinarian before the start of the study and preterminally. Ophthalmoscopy and auscultation of the chest were undertaken before the start of the study and before termination. Body weights were recorded weekly and food consumption was measured daily. Blood was taken for clinical pathology before the start of treatment, and after 3 and 5 weeks of treatment. Urine analysis was carried out. Lungs and kidneys were weighed at necropsy, and these organs and portions of other selected organs were processed for histopathological examination. There were no adverse clinical effects, nor were there any paraquat-related effects on ophthalmoscopy. On auscultation, increased respiratory sounds were heard in animals from several groups: of these, the finding in two males and three females at 90mg/kg may have been test material-related. Body weights decreased in males at a dietary concentration of 90mg/kg throughout the study and in the females at 90mg/kg towards the end of the study. Body-weight gain was reduced in those females given paraquat at 0.75mg/kgbw per day. Food intake was reduced at 90mg/kg in females towards the end of the study. In the males fed paraquat at a dietary concentration of 90mg/kg, there was a reduction in erythrocyte volume fraction, haemoglobin and erythrocyte count. No test material-related findings were seen in clinical chemistry investigations or urine analysis. One female fed the diet containing paraquat at 90mg/kg had a markedly increased lung weight. Changes were seen at 0.75mg/kgbw per day and at 90mg/kg in the lungs, both macroscopically and microscopically. The macroscopic changes comprised grey, red or purple depressed areas. In all animals receiving capsules containing paraquat at 0.75mg/kg bw per day, and in five of the six animals receiving diet containing paraquat at a concentration of 90mg/kg, there was histopathological evidence of alveolitis, such as intra-alveolar accumulations of mononuclear cells, interstitial hypercellularity and fibrosis and alveolar hyperplasia. Occasional giant cells and pigmented macrophages were seen. It was concluded that for paraquat administered in the diet the NOAEL was 35mg/kg (equivalent to 0.875mg of paraquat ion/kgbw per day) and that paraquat was more toxic when administered by capsule than when mixed with the diet (Sheppard, 1981a).

In a 13-week study, groups of three male and three female beagle dogs received paraquat (paraquat ion, 32.2% w/w) at a dietary concentration of 0, 7, 20, 60 or 120mg/kg as paraquat ion. These dietary concentrations are equal to doses of 0, 0.20, 0.55, 1.75 and

3.52 mg of paraquat ion/kgbw per day in males and 0, 0.24, 0.71, 1.92 and 4.26 mg of paraquat ion/kgbw per day in females. Animals were observed more than once daily, and by a veterinarian before the start of treatment and after 6 and 12 weeks of treatment. Ophthalmoscopy was carried out before the start of the study and after 6 weeks of treatment. Auscultation of the chest was carried out before the start of the study, 6 weeks after the start and immediately before the end of the study. The animals were weighed weekly and food consumption was measured daily. Blood samples were taken by jugular venepuncture before the start of treatment and after 6 and 12 weeks of treatment. These samples were used for haematological investigations and for clinical chemistry. At autopsy, selected organs were weighed and these and other selected organs were fixed and processed for histopathological examination. At the highest dietary concentration two males and two females were killed in extremis. These animals exhibited marked dyspnoea as well as increased respiratory sounds (harsh râles) and loss of body weight before they were killed. One female at 200 mg/kg showed pyrexia and malaise at 3 weeks; this was treated successfully with procaine penicillin and dihydrostreptomycin. The same animal showed loss of appetite from week 8; it was again treated with antibiotics. Survivors at the highest dose showed body-weight loss. Slight but significant reductions in weight gain were seen in females at 7, 20 and 60 mg/kg, compared with the controls; there was no clear dose-response relationship. These effects were not considered to be related to treatment. Food consumption was reduced in one female at the highest dietary concentration. Retinal engorgement was seen in one animal each at 7 mg/kg and 20 mg/kg, and two at 120 mg/kg. No intergroup treatment-related haematological or clinical chemistry findings were present, except in one of the decedents where haemoconcentration was seen. No test material-related effects on urinary parameters were seen. Absolute and relative lung weights were increased in all animals at 120 mg/kg and in two animals at 60 mg/kg; while not statistically significant (lungs from only two animals of each sex were weighed at the highest dietary concentration), these findings were considered to be biologically significant. Histopathological changes were seen in the lungs at 60 and 120 mg/kg. These changes consisted of proliferative alveolitis, with interstitial cellular infiltration (eosinophils and polymorphs) and exudate. Some renal (distal tubular) changes were seen in the same groups. The NOAEL was considered to be 20 mg/kg, equal to 0.55 mg/kg of paraquat ion per kg bw per day in males, and 0.71 mg/kg of paraquat ion per kg bw per day in females, on the basis of increases in lung weight and histopathological changes at the next higher dietary concentration (Sheppard, 1981b).

In a 1-year feeding study, groups of six male and six female beagle dogs were given diets containing technical-grade paraquat dichloride (paraquat ion, 32.2%) at a concentration of 0, 15, 30 or 50 mg/kg as paraquat ion for 1 year. Although no overall means were given in the study report, they were quoted in the submission document. Intakes were 0, 0.45, 0.93 and 1.51 mg of paraquat ion/kgbw per day for males and 0, 0.48, 1.00 and 1.58 mg of paraquat ion/kgbw per day for females (see Clapp, 2002). The dogs were observed twice daily, and examined by a veterinarian before the start of the study and after 13, 26 and 39 weeks, and also between weeks 48 and 51 of treatment; the examination by the veterinarian included auscultation and ophthalmoscopy. Body weights were measured weekly and food consumption daily. Haematology and clinical chemistry measurements were carried out during the study on jugular venous blood samples taken before the start of the study and at weeks 4, 8, 12, 16, 20, 39 and 52. Urine for urine analysis was collected over an 18 h period before the start of the study, and at weeks 8, 16, 24, 39 and 50. Urine samples were collected at week 39 for analysis for paraquat. At termination, necropsy was undertaken and selected organs were weighed, and these and other organs were processed for

histopathological examination. Samples of kidney, liver and lung, taken at necropsy, were analysed for paraquat. Respiratory dysfunction was observed at 50 mg/kg (hyperpnea). Increased vesicular sounds were heard in the lungs at auscultation. Erythema of the dorsum of the tongue was seen at 30 and 50 mg/kg in males, and at 50 mg/kg in females. No test material-related effects were seen on ophthalmoscopy. No test material-related effect on body-weight gain was seen. Reduced food consumption was seen at 50 mg/kg. No haematological changes were seen that were attributable to paraquat. Alkaline phosphatase activity was elevated in females at 30 and 50 mg/kg, and plasma concentrations of triglycerides were raised in both sexes at 50 mg/kg. Urinary specific gravity was elevated at 50 mg/kg in males. Lung weights (both absolute and relative) were significantly increased at 50 mg/kg. Spleen weights were elevated at 50 mg/kg in both sexes, but the biological significance was unclear, and the mean in males was influenced by one outlier. At 30 and 50 mg/kg, macroscopically there was yellow discoloration in the lungs. Microscopically, there was peribronchial mononuclear infiltration, peribronchial and interalveolar fibrosis and changes in the alveolar epithelium (alveolar cell hyperplasia and hypertrophy). These changes were accompanied by the presence of haemosiderin-laden macrophages. These changes were more severe at 50 mg/kg than at 30 mg/kg. Erythrophagocytosis in the bronchial lymph nodes was present at 30 mg/kg and 50 mg/kg. A dose-related increase in urinary paraquat was found at week 29. Paraquat was not found in the livers at any dietary concentration, but was found in the kidneys at 30 and 50 mg/kg. Paraquat was detected in the lungs. The NOAEL for the study was 15 mg/kg on the basis of erythema of the tongue at 30 mg/kg in males, elevated alkaline phosphatase in both sexes, and histopathological changes in the lung at ≥ 30 mg/kg. This NOAEL is equal to 0.45 mg of paraquat ion/kgbw per day (Kalinowski et al., 1983).

Cows

Groups of two Friesian cows were fed diets containing paraquat (as residues in dried grass) at a concentration of 25, 80 or 170 mg/kg as paraquat ion for 3 months. These dietary concentrations were equivalent to 0.375, 1.2 and 2.55 mg of paraquat ion/kgbw per day. During the trial, milk was collected from the cows. After they had been slaughtered, autopsy was carried out and organs, inter alia lung, liver and kidney, were examined histopathologically. Concentrations of paraquat were determined in the liver, kidney, renal fat and the pectoralis and adductor muscles. No adverse clinical effects were noted during the study, although the milk yield decreased (this was attributed to poor nutrition). No histopathological change attributable to paraquat was found. The concentration of paraquat in the milk was very low (in one sample, 0.001 mg/kg; in the remainder, <0.0006 mg/kg). The highest tissue residues were in the kidney (0.20–0.31) and liver (<0.01 –0.09). Concentration in cardiac and skeletal muscle and renal fat were much lower. The NOAEL was the highest dietary concentration, 170 mg/kg, equivalent to 2.55 mg of paraquat ion/kgbw per day (Edwards et al., 1974).

(b) Dermal application

Rabbits

In a 21-day study of dermal toxicity, groups of six male and six female New Zealand white albino rabbits were given technical-grade paraquat (purity, 33.5%), at a dose of 0, 1.5, 3.4, 7.8 or 17.9 mg/kgbw per day (equal to 0, 0.5, 1.15, 2.6 and 6.0 mg of paraquat ion/kgbw per day), applied in distilled water under an occlusive dressing to the clipped dorsal thorax. Distilled water without paraquat was applied to the control animals. The

period of exposure was 6 h per day. Animals were observed twice daily. They were more thoroughly examined and dermal irritation was assessed on days 1, 2, 4, 8, 11, 15, 18 and 21. Animals were weighed twice weekly and food consumption was measured weekly. Blood samples for haematology and clinical chemistry were collected before the start of the study and at termination. After 21 days, the animals were weighed and killed, and designated organs were weighed. These and further selected tissues were fixed and processed for microscopic pathological examination. No mortality was observed and all animals appeared to be clinically normal throughout the study. Body weights and food consumption were similar in all groups. No differences between the groups were seen in haematological measurements or clinical chemistry. Neither organ weight data nor histopathological examination showed evidence of test material-related systemic toxicity. Evidence of skin irritation was seen at the two highest doses. Microscopic evidence of skin irritation was seen in most animals at the highest dose and in some animals at a dose of 2.6 mg of paraquat ion/kgbw per day. Findings included erythema, erosion, ulceration, exudate, acanthosis and chronic inflammatory change. Accordingly, the NOAEL was 1.15 mg of paraquat ion/kgbw per day on the basis of skin changes at higher doses (Cox, 1986).

(c) *Exposure by inhalation*

Rats

In a 3-week inhalation study, an aerosol of technical-grade paraquat (paraquat ion, about 40%) was administered to groups of 36 male and 32 female albino Sprague-Dawley CD rats. The rats were exposed for 6 h per day, 5 days per week, for 3 weeks (i.e. 15 exposures). There were two control groups, one of which received no exposure to aerosol and the other received a saline aerosol. There were two test groups, one of which received aerosolized paraquat at a concentration of 0.01 µg of paraquat ion/l and the other 0.1 µg of paraquat ion/l. Particles had aerodynamic diameters of <0.7 µm. The rats were examined twice daily and, more thoroughly, once a week. Animals were weighed daily for the first week and then twice per week. Food consumption was measured weekly. Water consumption was measured daily, 5 days per week. Interim kills were carried out as follows: 3 days after the first exposure, four males and four females in each group were killed for histopathological examination of the nasal passages, pharynx, larynx and lungs (i.e. the rats were given a single exposure, left for 2 days, and then sacrificed). Additionally, 1 day after the third exposure, four males in each group were killed for examination of the nasal turbinates only. Eight animals of each sex per group were killed after the last exposure and the remainder (16 animals of each sex per group) were killed after a 3-week recovery period. Macroscopic examination was carried out post mortem but no microscopic pathology was performed. No treatment-related clinical signs were seen. No treatment had any effect on body-weight gain or food or water consumption. Aerosol containing paraquat ion at a concentration of 0.01 µg/l did not produce histopathological changes in the larynx, while exposure to aerosol containing paraquat ion at a concentration of 0.1 µg/l did produce such changes. In the animals examined 3 days after exposure at 0.1 µg of paraquat ion/l, there was squamous metaplasia at the base of the epiglottis. One day after the third exposure, there was ulceration, necrosis, acute inflammatory change and squamous metaplasia and/or hyperplasia especially at the base of the epiglottis and arytenoid processes. In those animals sacrificed in the interim for examination of the turbinates, no abnormalities were seen. Accordingly, the NOAEL for the study was 0.01 µg of paraquat ion/l on the basis of histopathological changes in the upper respiratory tract at the higher dose (Grimshaw et al., 1979).

2.3 Long-term studies of toxicity and carcinogenicity

Rats

In a 104-week study, groups of 80 male and 80 female Fischer (F344/DuCrj) rats were given diets containing paraquat dichloride (purity, >98%) at a concentration of 0, 10, 30, 100 or 300 mg/kg. Intakes of paraquat dichloride were 0, 0.35, 1.06, 3.52 and 10.6 mg/kg bw per day for males, and 0, 0.43, 1.34, 4.32 and 11.7 mg/kg bw per day for females. These intakes of paraquat dichloride represented intakes of 0, 0.26, 0.77, 2.55 and 7.67 mg of paraquat ion/kg bw per day in males, and 0, 0.31, 0.97, 3.13 and 8.47 mg of paraquat ion/kg bw per day in females. Eight rats of each sex were killed at 26, 52 and 78 weeks, while the survivors were sacrificed at 104 weeks. During the study, animals were observed daily and clinical findings, including mortality, were recorded. Animals that died during the study were subjected to necropsy followed by histopathological examination, as were those that were sacrificed in extremis. Body weight was measured weekly until week 26 of the study, and fortnightly thereafter. Food and water consumption was measured twice per week. At termination, haematological and clinical chemistry studies were carried out on 10 males and 10 females per group. At necropsy, selected organs were weighed and portions of these and of other organs were fixed and processed for histopathological examination. No clinical effect attributable to the test material was seen, but there was some indication of increased mortality between weeks 66 and 74 of the study in females at the highest dose. There was a reduction in body-weight gain and food and water consumption in both sexes at 300 mg/kg (the highest dietary concentration). The effect on body-weight gain was greater in the males. Some effects on haematology were observed. At 26 weeks, there was a decrease in white blood cell count at 300 mg/kg in males, but no differences between groups were seen in females. At 52 weeks, there were minor changes in mean corpuscular haemoglobin and haemoglobin concentration, and a decrease in white blood cell count at 300 mg/kg in males, but no differences between groups were seen in females. At 78 weeks, there was a decrease in white blood cell count at 300 mg/kg in males, but no test material-related differences between groups were seen in females. At 104 weeks, in males, there were minor changes in mean corpuscular volume and mean corpuscular haemoglobin. In females, at 104 weeks, there were minor changes in mean corpuscular haemoglobin concentration (a reduction) at 300 mg/kg. At 26 weeks, in males, a reduction in aspartate aminotransferase activity and globulin was observed at 300 mg/kg, as well as a rise in blood concentrations of glucose at 100 and 300 mg/kg. At 26 weeks, in females, an increase was seen in γ -glutamyl transpeptidase at 300 mg/kg, and a decrease at 10 mg/kg. At 26 weeks, total protein, albumin and globulin concentrations were all decreased in females at 300 mg/kg. At 52 weeks, in males, a reduction in aspartate aminotransferase, alanine aminotransferase and γ -glutamyl transpeptidase activity, and in cholesterol and calcium concentrations was seen at 300 mg/kg. At 52 weeks, females showed no test material-related changes in clinical chemistry. At 78 weeks, males showed reductions in alkaline phosphatase, alanine aminotransferase and γ -glutamyl transpeptidase activity were seen accompanied by an increase in albumin and a decrease in globulin at 300 mg/kg, whereas females showed no test material-related changes in clinical chemistry. At termination at 104 weeks, a decrease in globulin was seen in males at 300 mg/kg, while no noteworthy changes in clinical chemistry parameters were seen in females. In males at the highest dietary concentration, body weight at necropsy was decreased at 26, 52 and 78 weeks and at termination. Although some changes in organ weights were observed, many of these did not appear to be test material-related. At 26 weeks in males at 300 mg/kg, however, relative but not absolute lung weight was increased, as it was at 52 weeks. At 300 mg/kg, at 78 weeks, absolute lung weight was decreased and relative lung weight in males did not differ from those of controls, while at

termination, neither value was different from that of controls. In females, at 26 weeks, a reduction in body weight was not seen at any concentration. At 26 weeks, an increase in relative lung weight was seen at the two higher dietary concentrations, and this was accompanied by an increase in absolute lung weight at 100 mg/kg only. At 52 weeks and 78 weeks, there were no differences between groups in body weight or absolute or relative lung weight in females. At termination, in females, decreased body weight and an increased relative but not absolute lung weight was observed at 300 mg/kg. A reduction in absolute and relative ovarian weight was observed at 26 weeks at the highest dietary concentration. On histopathological examination, there were changes in the lungs at 300 mg/kg in both sexes and at 100 mg/kg in males. The changes consisted of proliferation of interalveolar septum cells and hyperplasia of the alveolar epithelium. The frequency of pulmonary adenoma was increased in females at 300 mg/kg (see Table 2). Histopathological evidence of cataract was found in both sexes at 300 mg/kg (see Table 3). The NOAEL for the study was 30 mg/kg (1.06 mg of paraquat dichloride/kg bw per day and 1.34 mg of paraquat dichloride/kg bw per day in males and females respectively) on the basis of clinical chemistry changes in males, increased lung weight in females and histopathological changes in the lungs of males at ≥ 100 mg/kg. These NOAELs are equal to 0.77 and 0.97 mg of paraquat ion/kg bw per day in males and females respectively (Yoshida et al., 1982).

In a study of chronic toxicity, groups of 62 male and 62 female JCL:Wistar rats were fed diets containing paraquat (purity, 98%) at a concentration of 0, 6, 30, 100 or 300 mg/kg for up to 104 weeks. These dietary concentrations provided intakes of paraquat dichloride equal to 0, 0.25, 1.26, 4.15 and 12.25 mg/kgbw per day in males, and 0, 0.3, 1.5, 5.12, 15.29 mg/kgbw per day in females. These intakes are equal to 0, 0.18, 0.91, 3.00 and 8.87 mg of paraquat ion/kgbw per day in males, and 0, 0.22, 1.09, 3.71 and 11.1 mg of paraquat ion/kgbw per day in females. Six rats of each sex per group were killed at 26 weeks and 52 weeks; the survivors were killed at 104 weeks. The rats were examined twice per day, deaths were recorded and clinical findings noted. Ophthalmoscopy was carried out before treatment, and before sacrifice in those killed at 26 and 52 weeks, and at termination. Body

Table 2. Incidence of lung tumours in rats fed diets containing paraquat (survivors + decedents)

Sex	Lung tumour	Dietary concentration (mg/kg)				
		0	10	30	100	300
Males	Adenoma	1	2	3	4	3
	Adenocarcinoma	0	0	2	1	3
Females	Adenoma	1	2	0	1	7
	Adenocarcinoma	0	0	0	0	0

From Yoshida et al., (1982)

Table 3. Incidence of cataract in rats fed diets containing paraquat (decedents + survivors)

		Dietary concentration (mg/kg)				
		0	10	30	100	300
Males		8	4	7	9	46
Females		7	7	8	11	42

From Yoshida et al. (1982)

weight and food consumption were measured weekly until week 26 and thence fortnightly. Haematological and clinical chemistry end-points were measured in blood samples taken from animals killed at 26 weeks, at 52 weeks, and from the survivors at termination. Included among the clinical chemical parameters measured were activities of plasma, erythrocyte and brain cholinesterases. Urine analysis was performed on the animals killed at 26 and 52 weeks and on the survivors at termination. Animals killed at 26 and 52 weeks and survivors to termination were subjected to necropsy, as were decedents. Selected organs were weighed and these and other organs were fixed and processed for histopathological examination. No clinical effects were observed. At the highest dietary concentration in females, there was a decrease in weight gain in the middle of the study (weeks 43, 42–48 and 54), otherwise body-weight gain was unaffected. No substantial intergroup differences in food consumption or in water intake were noted. At week 26, at the highest dietary concentration, there was a decrease in erythrocyte count, erythrocyte volume fraction and haemoglobin and a reticulocytosis in males and in the erythrocyte count and haemoglobin in females. At 300 mg/kg, at week 52, decreased erythrocyte count and increased numbers of polymorphs were seen in males, and lowered erythrocyte count, haemoglobin concentration and leukocytes were seen in females. At week 104, both sexes showed decreases in erythrocyte count, erythrocyte volume fraction and haemoglobin, and an increase in reticulocytes was observed in males. At 26 weeks, a decrease in total protein was seen in both sexes at 300 mg/kg, with a decrease in alkaline phosphatase activity in females at this dietary concentration. At week 52, decreased total protein was found in both sexes, as well as reduced blood concentrations of glucose in males and reduced aspartate aminotransferase and alanine aminotransferase activities in females. There were no differences between the groups in urine analysis at any time-point. In the rats sacrificed at week 26, there were increases in absolute kidney weights (right kidney only) in males and in both absolute kidney weights in females and absolute ovarian weights in females. At week 52, at the highest dietary concentration, in males there was an increase in both absolute thyroid and kidney weights, while females showed an increase in absolute ovarian weights and a decrease in the relative weights of the brain, heart and liver. At termination, at 300 mg/kg, males showed reductions in the absolute and relative heart weights, while females showed lowered absolute and relative liver weights and decreased absolute heart weight. At necropsy and histopathological examination, no findings could be attributed to the test material. The NOAEL was therefore 100 mg/kg in both sexes (equal to 4.15 and 5.12 mg of paraquat dichloride/kg bw per day in males and females respectively), on the basis of haematological observations and lowered plasma concentration of total protein at the highest dietary concentration. These NOAELs are equal 3.00 and 3.71 mg of paraquat ion/kg bw per day in males and females, respectively (Toyoshima et al., 1982).

Groups of 70 male and 70 female Fischer 344 rats were given diets containing paraquat (technical grade, 32.69%) at a dietary concentration of 25, 75 or 150 mg/kg as paraquat ion (equivalent to a dose of 1.25, 3.75 or 7.5 mg of paraquat ion/kg bw per day) for a period of at least 113 weeks (males) and 122 weeks (females). Two additional groups of rats served as controls. There were also additional satellite groups of five males and five females from one control group and all three test groups which were sacrificed at 1 year for estimation of paraquat concentrations in certain tissues. Ten male and ten female rats from each group were sacrificed for histopathological examination at 1 year. Rats were inspected once or twice daily, mortality was recorded and rats in extremis were sacrificed and necropsied (see below). Ophthalmoscopic examination of both eyes was carried out before the start of the study and after 4, 14, 26, 52 and 79 weeks of treatment for 20 males and 20 females from each control group; the test groups were examined in a similar manner.

Surviving males were examined ophthalmoscopically at 110 weeks and 112/113 weeks (termination) and surviving females at 110 and 118/119 (termination). Food consumption was recorded weekly and water consumption was recorded over 3-day periods during each of the first four weeks, and during weeks 13, 26, 41, 52, 65, 78, 92 and 101. Body weight was recorded weekly for the first 12 weeks, then fortnightly until week 68, and then weekly until termination. Before the start of treatment and after 14, 26, 40, 53, 66, 79, 92 and 102 weeks, blood was taken for measurement of haematological and clinical chemistry parameters, and additionally in males at 111/112 weeks and in females at 118/119 weeks. Urine samples were collected periodically for urine analysis and for analysis of paraquat in the urine. Five animals of each sex per group were sacrificed at 52 weeks for estimation of concentrations of paraquat in the liver, lungs, kidneys, skin, plasma and urine. Necropsy was performed on all decedents, the 10 animals of each sex per group sacrificed at 52 weeks and those surviving to termination, and selected organs were weighed. These and other selected organs were preserved and processed for histopathological examination. Mortality was not affected by treatment and survival to termination was 38–55% in males and 47–50% in females. No clinical adverse effect was seen, except corneal opacity, which was seen at 150mg/kg in males and at 75mg/kg in females. At ophthalmoscopy, cataracts were seen at 150mg/kg in both sexes and, from 103 weeks, at 75mg/kg in both sexes. In the males, the prevalence of cataracts was not unequivocally increased at 25mg/kg; however, a statistical analysis of the eye changes revealed an increase in posterior capsular changes at week 110 in the males at 25mg/kg. Food intake at 150mg/kg was reduced in both sexes, in the males for the first year of the study and in the females during the first 6 weeks; these changes were small. Depression of body-weight gain was seen at 150mg/kg in both sexes, but was more severe in males and was also present in males at 75mg/kg. Body-weight gain in males at 25mg/kg and in females at 75 and 25mg/kg was not different from that in the controls. Test material-related effects were not seen on haematological or clinical chemistry parameters, or on urine analysis. At 52 weeks, the concentration of paraquat in the urine was dose-related. In rats sacrificed at 52 weeks, paraquat was detected in the plasma and kidneys at all dietary concentrations, while paraquat was present in the lungs of animals at 75 and 150mg/kg; only at 150mg/kg and in females was paraquat found in the liver. Paraquat was found in some skin samples taken from males at 75mg/kg and from both sexes at 150mg/kg. No test material-related effects were seen on organ weights, other than those attributable to changes in body weight. Macroscopically, there was an increase in corneal opacity and focal sub-pleural changes at 75 and 150mg/kg. Proliferative alveolar changes were also seen at these dietary concentrations. Lung histopathology was examined by two groups (Tables 4–6). An

Table 4. Initial assessment of lung histopathology in rats given diets containing paraquat

	Dietary concentration (mg/kg)									
	0 (Control group 1)		0 (Control group 2)		25		75		150	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Number of rats examined	70	70	69	69	70	70	70	70	69	70
Adenoma	1	0	2	0	3	1	5	2	4	8 ^a
Carcinoma ^b	1	0	0	0	1	1	1	1	3	2
Total neoplasia	2	0	2	0	4	2	6	3	7	10 ^a
Alveolar epithelialization	2	3	2	7	2	5	7	8	8	3

From Woolsgrove (1983)
^a*p* < 0.001
^b Bronchiolar-alveolar or squamous cell carcinomas

Table 5. Second assessment of lung histopathology in rats given diets containing paraquat

	Dietary concentration (mg/kg)									
	0 (Control group 1)		0 (Control group 2)		25		75		150	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Number of rats examined	70	70	69	69	70	70	70	70	69	70
Adenoma	0	0	0	0	2	0	1	1	1	0
Carcinoma	1	0	1	0	2	1	1	1	3	0
Adenomatosis	2	4	4	4	5	5	8	4	11 ^a	13 ^a

From Ishmael & Godley (1983)
^a*p* < 0.01

Table 6. Final assessment of lung histopathology in rats given diets containing paraquat

	Dietary concentration (mg/kg)									
	0 (Control group 1)		0 (Control group 2)		25		75		150	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Number of rats examined	70	70	69	69	70	70	70	70	69	70
Bronchioalveolar adenoma	2	0	0	0	2	0	0	1	0	1
Bronchioalveolar carcinoma	1	0	1	0	2	1	2	1	2	1
Squamous cell carcinoma	0	0	0	0	0	0	1	0	2	0
Focal adenomatous hyperplasia	2	4	3	5	7	5	9	7	15	7
Diffuse adenomatous hyperplasia	0	1	0	0	0	0	0	0	1	3
Focal alveolar wall fibrosis	1	8	4	5	4	8	6	13	3	12
Diffuse alveolar wall fibrosis	0	3	0	5	2	3	3	4	8	3

From Busey (1986)
^a*p* < 0.01

initial assessment of lung histopathology was made based on that of Life Sciences Research's own staff pathologists and of two consultant pathologists from the USA (Table 4). The other assessment was by Ishmael (Table 5), at that time Head of Pathology at Imperial Chemical Industries. There were some clear differences. Finally, the slides were examined by four independent pathologists and the results were reported by Busey (1986) (Table 6). It was concluded that the differentiation of bronchiolar adenomas and carcinomas from the non-neoplastic lesions typical of paraquat was difficult. However it was also concluded that the incidence of lung neoplasms in the test groups was comparable to that in the control groups (Ishmael & Godley, 1983; Woolsgrove, 1983; Sotheran et al., 1981; Woolsgrove, 1985; Busey, 1986; Ishmael, 1987).

It was concluded from the data summarized in Table 6 that there was no association between the incidence of adenomas, carcinomas or the two combined, and exposure to paraquat. In contrast, there was a significantly increased incidence of adenomatosis at 150mg/kg when all animals were included in the analysis (i.e. those sacrificed at 52 weeks, decedents and those killed at termination).

Ishmael (1987) reviewed the slides of the head region, in which squamous cell carcinomas of the skin and subcutis had been reported. In males, 11 such tumours were seen in the study (1, 2, 2, 0 and 6 in the two control groups and at the lowest, intermediate and

highest doses, respectively) as originally reported and in Ishmael (1987). The site of origin of these tumours, however, differed and Ishmael (1987) suggested they should not be considered as a single phenomenon for statistical purposes. Other changes seen included dilatation of the fourth ventricle of the brain (hydrocephalus) in females at 75 and 150mg/kg. Cysts and cystic spaces were seen in the spinal cords and, in males, prevalence was significantly greater than that in the controls in all test groups, although there was no clear dose-response relationship. This pathological change was found in females, but the frequency in test groups and control groups was similar (and similar to the frequency in the males in test groups). Degeneration of the sciatic nerve was found in males at 75 and 150mg/kg. Changes were present in the eyes. At the highest doses, peripheral lenticular degeneration, more severe in females, and pear-shaped posterior peripheral lenticular change was seen. Mid-zonal lenticular degeneration, lens capsular fibrosis and/or lens ruptures were all seen. At 75mg/kg, changes were milder. These changes were seen in both decedents and those rats surviving to termination. At the highest concentration, in the decedents, peripheral retinal degeneration was observed in females and proteinaceous vitreous humour was seen in males. Some changes were seen at the lowest dietary concentration; in male survivors these were moderate peripheral morgagnian corpuscles, slight peripheral lenticular degeneration, moderate mid-zonal lenticular degeneration and loss of outer nuclear layer of the retina. The last was unlikely to be a compound-related effect as the prevalence was lower in both the controls and at higher doses. In female survivors to termination, at the lowest dietary concentration, changes observed were moderate peripheral morgagnian corpuscles, slight peripheral lenticular degeneration and moderate mid-zonal lenticular degeneration.

At termination, there was no clear evidence of an effect on the retina at the lowest dose, although in males at the two higher dietary concentrations there may have been an effect on the periphery of the retina. This study was continued for a longer duration than that recommended by the OECD (104 weeks for long-term studies in rats). The NOAEL was 25mg/kg for lenticular lesions after 103 weeks at 25mg/kg in males and likewise in females at 103 and 110 weeks (see Table 7 for ophthalmoscopy findings at 103 weeks and Table 8 for lens findings at necropsy). This NOAEL is equivalent to 1.25mg of paraquat ion/kgbw per day. This interpretation is supported by the findings from the other long-term studies in rats.

Table 7. Frequency of effects on the lens (in life) at 103 weeks in rats given diets containing paraquat

Finding	Dietary concentration (mg/kg)									
	0 (Control group 1)		0 (Control group 2)		25		75		150	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Opacity	1	0	4	0	0	0	0	1	0	0
Vacuolation	0	0	1	0	0	0	0	0	1	1
Suture line opacity	0	1	1	0	1	0	14*	9*	1	1
Posterior polar opacity/cataract	3	0	0	0	1	0	8	5	19	30
Posterior capsular opacity/cataract	0	2	0	5	0	4	3*	6*	24*	12*
Radial cataract	0	0	0	0	1	0	2	2*	8*	5*
Total cataract	1	1	1	1	2	1	3	1	5*	4

From Ishmael (1987)
* Greater incidence than combined control groups, statistically significant at $p = 0.05$ or less

Table 8. Frequency of effects on the lens at necropsy in rats given diets containing paraquat (all animals, regardless of time of death)

Finding	Dietary concentration (mg/kg)							
	0 (Control groups 1 and 2)		25		75		150	
	Males	Females	Males	Females	Males	Females	Males	Females
Number of eyes examined	219	226	112	112	114	107	115	114
Peripheral morgagnian corpuscles								
Slight	64	50	29	14	26	8*	7*	8*
Moderate	38	70	31	39	19	27	25	16
Marked	12	34	19*	31*	35*	52*	69*	84*
Peripheral lenticular degeneration								
Slight	18	60	25*	29	32*	23	26*	10*
Moderate	8	33	13*	30*	39*	31*	34*	43*
Marked	1	7	4	4	6*	10*	22*	32*
Pear-shaped posterior lenticular change	6	42	11*	32*	51*	48*	73*	74*
Midzonal lenticular degeneration								
Slight	7	27	5	20	18*	14	14*	19
Moderate	0	12	4*	13	19*	27*	39*	37*
Marked	0	0	0	3	3*	23*	29*	27*
Heart-shaped	0	2	0	0	1	18*	18*	15*

From Ishmael (1987)

* Greater incidence than combined control groups, statistically significant at $p = 0.05$ or less

Mice

In a 104-week study, groups of 80 male and 80 female JCL:ICR mice were given diets containing paraquat (paraquat dichloride; purity, 98%) at a dietary concentration of 0, 2, 10, 30 or 100 mg/kg, providing intakes of paraquat dichloride equal to 0, 0.26, 1.31, 3.92 and 13.09 mg/kg bw per day in males, and 0, 0.26, 1.32, 3.82 and 13.03 mg/kg bw per day in females. These intakes are equal to 0, 0.19, 0.95, 2.84 and 9.48 mg of paraquat ion/kg bw per day in males, and 0, 0.19, 0.96, 2.77 and 9.43 mg of paraquat ion/kg bw per day in females. At weeks 26 and 52, 10 male and 10 female per group were sacrificed. The mice were examined twice daily and adverse clinical effects, including mortality, were noted. Body weight and food consumption were measured weekly until week 26, and fortnightly thereafter. Blood was taken for haematology and clinical chemistry (including determination of plasma, erythrocyte and brain cholinesterase activities) from the animals killed at 26 and 52 weeks and from those that survived to termination. Urine analysis was performed on animals killed at 26, 52 and 104 weeks. Survivors were sacrificed at 104 weeks. Necropsy was carried out on the animals killed at 26 and 52 weeks and on those that survived to termination, as well as the decedents. Selected organs were weighed and tissue from these and further selected organs was fixed and processed for histopathological examination. There were no effects of the test material on mortality. No clinical effects attributable to the test material were noted. The test material had no effect on body-weight gain or food consumption. Falls in the erythrocyte count, erythrocyte volume fraction, haemoglobin, white blood cell count and lymphocyte count were noted in males and in the haemoglobin concentration and white blood cell count in females at 100 mg/kg in week 26. At week 52, also at 100 mg/kg, a decreased erythrocyte count, and decreases in erythrocyte volume fraction and white blood cell count were observed in males and lowered erythrocyte count and

haemoglobin concentration in females. At week 104, lowered erythrocyte count, erythrocyte volume fraction and polymorphonuclear leukocytes (%) were observed in males, and decreases in the erythrocyte volume fraction and haemoglobin concentration in females. Clinical chemistry findings included lowered total plasma protein in both sexes at the highest dietary concentration in week 26. At week 52, lowered total protein was seen in males and decreases in aspartate aminotransferase and alkaline phosphatase activities, with increased blood concentration of glucose, were seen in females. At week 104, reductions in total protein and increases in blood glucose were observed in both sexes. Urine analysis showed no abnormality at any time in either sex. The absolute and relative weights of the (left) adrenal at 30 mg/kg in males killed at 26 weeks was decreased in comparison with those of the controls. In male at 100 mg/kg, at 26 weeks, adrenal and thyroid absolute and relative weights were decreased in comparison with those of controls, and absolute and relative lung weights were elevated. At week 52, there was an increase in absolute heart weight in males, while at week 104, decreases in absolute thyroid, liver and bladder weight were noted in males, together with an increase in relative (left) kidney weight. A drop in the absolute brain weight was noted at week 104 in females. No macroscopic or microscopic changes that could be attributed to the test material were found in the decedents or sacrificed animals. The NOAEL was therefore 30 mg/kg on the basis of haematological and clinical chemistry changes in both sexes, at the next highest dietary concentration. This was equal to 3.92 and 3.82 mg of paraquat dichloride/kg bw per day in males and females, respectively (2.84 and 2.77 mg of paraquat ion/kg bw per day in males and females, respectively) (Toyoshima et al., 1982).

A lifetime feeding study in mice was carried out; termination was at 97–99 weeks, at which time mortality was approaching 80%. Groups of 60 male and 60 female Swiss mice were fed diets containing paraquat at a dietary concentration of 0, 12.5, 37.5 or 100 mg/kg (technical grade dichloride; paraquat ion, 32.7%) for up to 99 weeks. The dietary concentration of paraquat received by groups of mice at 100 mg/kg was increased to 125 mg/kg from week 36, as few adverse effects had been noted, other than a decrease in food consumption, up to that time. The intakes of test material were equivalent to 0, 1.88, 5.62 and 15.0/18.7 mg of paraquat ion/kg bw per day. The control groups were duplicated. Satellite groups of 10 male and 10 female mice received the diet for 52 weeks and were used for measurement of paraquat concentrations in plasma, kidney and lung; in the case of the satellite groups, the control groups were not duplicated. Further satellite groups of 15 mice of each sex were fed the diet and acted as microbiological sentinels. The mice were observed daily for clinical effects, while body-weight determinations were recorded weekly for 12 weeks, fortnightly from week 12–36, weekly from week 36–40 and thence fortnightly. Food consumption measurements were undertaken weekly for the first 12 weeks of the study, and during weeks 36–40; at all other times, it was measured for 1 week during each 4-week period. Urine samples for measurement of concentration of paraquat were collected at 3-month intervals. Mice in extremis were sacrificed, as were those that survived to termination. These mice and decedents were subjected to necropsy, after which histopathological examination was carried out. Tissues from the satellite group were not subjected to autopsy. The main clinical findings were swellings and sores in the genital area of the male and, to a lesser extent, female mice, accompanied by incontinence. Mortality was greater than in the combined control groups in male mice receiving paraquat at a dietary concentration of 37.5 mg/kg and in female mice receiving paraquat at a dietary concentration of 125 mg/kg. The former is unlikely to be a compound-related effect, as an increase in the mortality at the highest dietary concentration was not observed in males. Body-weight gain was unaffected in males, while body-weight gain was decreased in females at the highest dietary

concentration (but not until this dietary concentration had been raised from 100 mg/kg to 125 mg/kg) and, after week 68, at a dietary concentration of 37.5 mg/kg. Food consumption was affected: in males, food consumption was reduced at all dietary concentrations to some extent, particularly early in the study; however, the effect did not appear to be dose-related. In females, decreased food consumption at all dietary concentrations was found, but this was more consistent and severe at the highest concentration. Concentrations of paraquat in the urine were found to be dose-related. On one occasion, paraquat was found in the urine of female controls, in trace amounts. In the satellite group, concentrations of paraquat in plasma were related to the dietary concentration that the animals had received for males, but less clearly so for females. In the case of the liver and lung tissue samples, some difficulties were encountered with some samples in analysis, but where analysis was possible, the results appeared to be related to dose. Changes in the proximal tubules of the kidneys (hydropic degeneration, eosinophilia, degeneration and/or dilatation) were seen at the highest dietary concentration, and there was evidence that, in the decedents, the predominant change was hydropic degeneration, eosinophilia, while degeneration and/or dilatation were seen in survivors to termination. Some very mild renal changes were seen in males at 37.5 mg/kg. In the lungs, alveolar focal hypercellularity was found at a higher frequency at the highest dietary concentration than at the lower concentrations or in controls in both sexes. There was no evidence of differences in cataract formation between the groups. There was an increase in adenomas in males and females receiving paraquat at the highest dietary concentration and dying after 52 weeks and before termination than in controls, but this was not dose-related. Moreover, at termination, a lower incidence of these tumours was observed in these animals than in controls. The NOAEL was 12.5 mg/kg (equivalent to 1.88 mg of paraquat ion/kg bw per day) on the basis of decreased body-weight gain in females and renal changes in males at the next highest dietary concentration. Paraquat was not considered to be tumorigenic (Sotheran et al., 1981).

2.4 Genotoxicity

Paraquat has been the subject of many tests for genotoxicity (see Table 9). Paraquat consistently gave negative results in well-established assays for reverse mutation in strains of *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98 and TA100). There was one positive result in *S. typhimurium* TA102, a strain that is particularly responsive to reactive oxygen species. More variable results were obtained in the less well-established assays for forward mutation in *S. typhimurium* and in assays for DNA damage in bacteria, for example the *umu* test, SOS test, tests for DNA repair and the rec assay) and in an assay for gene mutation in *Aspergillus nidulans*. In comparison with the assays for reverse mutation in *S. typhimurium*, these assays are not well validated. The assays for mutation in plants are not well validated and no conclusions could be drawn from them. Paraquat gave fairly consistently positive results in assays for chromosomal damage in mammalian cells. Positive results were consistently obtained in assays for DNA damage (sister chromatid exchange, unscheduled DNA synthesis and the comet assay) in mammalian cells. These data suggest that paraquat has mutagenic potential in vitro.

The results of tests for genotoxicity with paraquat in *Drosophila melanogaster* were conflicting, and are in any case irrelevant to the situation in mammals in vivo. In studies of DNA damage (unscheduled DNA synthesis) in mammalian systems in vivo and of chromosome damage in germ cells (dominant lethal test), paraquat gave negative results. The results of the majority of assays for clastogenicity (metaphase analysis to investigate chromosomal aberrations or tests for micronucleus formation) in bone marrow were negative.

Table 9. Results of studies of genotoxicity with paraquat

End-point	Test-object	Concentration/Dose	Purity	Results	Reference
<i>In vitro</i>					
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100	1–1000 µg/plate	>99.9%	Negative ± S9	McGregor (1977)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100	0.5–500 µg/plate	100%	Negative ± S9	Shirasu et al. (1978)
Reverse mutation	<i>S. typhimurium</i> , TA1535, TA1538, TA98, TA100	0.16–5000 µg/plate	>99%	Negative ± S9	Anderson (1977)
Reverse mutation	<i>E. coli</i> , WP2 <i>hcr</i>	0.5–500 µg/plate	100%	Negative ± S9	Shirasu et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100	1–50 µg/plate	NS	Negative	Benigni et al. (1979)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100	Not clear	NS	Negative	Eisenbeis et al. (1981)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	0–1 mmol/l	NS	Negative	Moody & Hassan (1982)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100 (not clear what other strains were used), <i>E coli</i> WP2 <i>hcr</i>	Not clear	NS	Negative, but full results not given	Shirasu et al. (1982)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100, <i>E coli</i> WP2 <i>hcr</i>	5000 µg/plate	NS	Negative	Moriya et al. (1983)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA97, TA98, TA100	0–20 µg/plate	NS	Negative	Lin et al. (1988)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA97, TA98, TA100	0–50 µg/plate	NS	Negative	Lin et al. (1989)
Reverse mutation	<i>S. typhimurium</i> TA102	10 ng/plate	NS	Negative	Levin et al. (1984)
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA1538, TA98, TA100 <i>E. coli</i> WP2 <i>hcr</i>	0.5–500 µg/plate	NS	Negative	Shirasu et al. (1978)
Reverse mutation	<i>E. coli</i> IC203, <i>oxyR</i> deficient and WP2 <i>uvrA</i> /Pkm101	1 µg/plate	NS	Negative	Martínez et al. (2000)
Reverse mutation	<i>S. typhimurium</i> TA100, TA98	0.06 µmol/plate	NS	Negative	Nishimura et al. (1982)
Reverse mutation	<i>S. typhimurium</i> TA102, TA2638, <i>E. coli</i> WP2/Pkm101 and WP2 <i>uvrA</i> /Pkm101	0–10 µg/plate	NS	Positive	Yamaguchi (1981)
Reverse mutation	<i>S. typhimurium</i> TA100	20 µg/plate	NS	Negative	Watanabe et al. (1998)
Forward mutation	Mouse lymphoma L5178Y cells <i>Tk</i> ^{+/−}	31.3–1000 µg/plate	45.66% w/w technical-grade paraquat dichloride	Negative ± S9	Clay & Thomas (1985)
Forward mutation at the <i>Hprt</i> locus	Chinese hamster V79 cells	1–5 mmol/l	NS	Negative	Speit et al. (1998)
Forward mutation to azaguanine resistance	<i>S. typhimurium</i> His G46, TA92, TA1535	0.1–1 µg/plate	NS	Positive	Benigni et al. (1979)

Table 9. Continued

End-point	Test-object	Concentration/Dose	Purity	Results	Reference
Forward mutation to azaguanine resistance	<i>S. typhimurium</i> His G46, TA92, TA1535, TA1538, TA100	0.1–2.5 µg/plate	NS	Positive	Bignami & Crebelli (1979)
<i>Umu</i> test	<i>S. typhimurium</i> TA1535/Psk1002	1000–3333 µg/ml	NS	Positive	Oda et al. (1985)
<i>Umu</i> test	<i>S. typhimurium</i> TA1535/Psk1002	1000 µg/ml	NS	Positive	Nakamura et al. (1987)
<i>Umu</i> test	<i>S. typhimurium</i> TA1535/Psk1002	0.1 ml/tube	NS	Negative	Degirmenci et al. (2000)
<i>Umu</i> test	<i>E. coli</i> K12 AB1157, AB2463 H/r30, H/s30, NG30, R15, B/r, B _{S-1}	4 mg/ml	NS	Positive	Degirmenci et al. (2000)
SOS-induced DNA damage	<i>E. coli</i> WP2 _s (λ)	0.02–67.11 µmol/l	NS	Positive ± S9	DeMarini & Lawrence (1992)
SOS-induced DNA damage	<i>E. coli</i> PQ300	Not clear	99	Negative	Eder et al. (1989)
SOS-induced DNA damage	<i>E. coli</i> PQ37, PM21, GC4798	Not clear	NS	Negative	Müller & Janz (1992)
DNA repair	<i>S. typhimurium</i> TA1538, TA1978	100 µg/plate	NS	Positive	Benigni et al. (1979)
Rec assay	<i>B. subtilis</i> recombination wild-type H17 and deficient M45	1–500 µg/disc	100%	Negative	Shirasu et al. (1978)
Gene mutation	<i>Aspergillus nidulans</i> (plate assay)	0–1000 µg/plate	NS	Positive	Benigni et al. (1979)
Gene mutation	<i>A. nidulans</i> (liquid assay)	20 mg/ml	NS	Negative	Benigni et al. (1979)
Lethal recessive	<i>A. nidulans</i> (liquid assay on quiescent conidia)	20 mg/ml	NS	Positive	Benigni et al. (1979)
Intrachromosomal recombination	<i>Saccharomyces cerevisiae</i>	0–35 mg/ml	NS	Negative	Brennan et al. (1994)
DNA damage	<i>S. cerevisiae</i>	1–20 mmol/l	NS	Negative	Paesi-Toresan et al. (1998)
Gene conversion	<i>S. cerevisiae</i>	100–900 mg/kg	NS	Positive	Parry (1973)
Gene conversion	<i>S. cerevisiae</i>	1000 mg/kg	NS	Negative	Siebert & Lemperle (1974)
Reverse and forward mutation	<i>Nostoc muscorum</i> (blue-green alga)	50 and 75 mg/kg	NS	Positive	Vaishampayan (1984a)
Reverse and forward mutation	<i>N. muscorum</i> (blue-green alga)	25–75 mg/kg	NS	Positive	Vaishampayan (1984b)
Cytogenetics	<i>Vicia fava</i> (broad/fava bean)	NS	NS	Negative	Gopalan & Njagi (1979)
Somatic mutation (<i>Drosophila</i> wing spot test)	<i>Drosophila melanogaster</i>	2–8 mmol/l	99%	Negative	Torres et al. (1992)
SMART assay	<i>D. melanogaster</i>	NS	NS	Negative	Ramel & Magnusson (1992)
SMART assay	<i>D. melanogaster</i>	0–10 mmol/l	NS	Negative	Gaivao & Comendador (1996)
SMART assay	<i>D. melanogaster</i>	0–10 mmol/l	NS	Positive	Gaivao et al. (1999)
SMART assay	<i>D. melanogaster</i>	0–16 mmol/l	NS	Negative	Vontas et al. (2001)

Table 9. Continued

End-point	Test-object	Concentration/Dose	Purity	Results	Reference
Chromosome test	<i>D. melanogaster</i> , <i>mus</i> 302 repair-defective females	200 mg/kg	NS	Negative	Woodruff et al. (1983)
Forward mutation at <i>Tk</i> locus	Mouse lymphoma L5178Y cells	0–200 µg/ml	NS	Positive	McGregor et al. (1988)
Mutation to thioguanine resistance	Chinese hamster V79 cell lines transfected with bacterial <i>gpt</i> (G12, G10)	200–300 µmol/l	NS	Negative	Kitahara et al. (1996)
Chromosomal aberration	Chinese hamster cells	≤200 µg/ml	45% technical grade	Positive	Lin et al. (1987)
Chromosomal aberration	Chinese hamster cells resistant to hydrogen peroxidized (H ₂ O ₂)	50–400 µg/ml	NS	Positive	Sawada et al. (1988)
Chromosomal aberrations and sister chromatid exchange	Chinese hamster fibroblast cells	3–10 mmol/l for chromosomal aberrations, and 0–0.75 mmol/l for sister chromatid exchanges	NS	Positive	Nicotera et al. (1985)
Chromosomal aberrations and sister chromatid exchange	Chinese hamster lung cells	0.08–20 µmol/l	NS	Positive	Tanaka & Amano (1989)
Chromosomal aberration	Human peripheral blood lymphocyte culture	1–50 µg/ml, chromosomal aberrations	99%	Negative	Ribas et al. (1997/8)
Sister chromatid exchanges	Chinese hamster ovary cells	0.625–100 µg/ml	45% technical grade	Negative	Wang et al. (1987)
Sister chromatid exchanges	Rat tracheal epithelial cells	0.625–2.5 µg/ml	45% technical grade	Positive	Wang et al. (1987)
Sister chromatid exchanges	Human peripheral blood lymphocyte culture	1–4000 µg/ml for sister	99%	Positive	Ribas et al. (1997/8)
Sister chromatid exchange	Chinese hamster lung fibroblasts	1.2–245 µg/ml	99.4% dichloride	Positive –S9, effect less +S9	Howard et al. (1985)
Cytogenetics	Human lymphocytes	250–2500 µg/ml	99.6% w/w dichloride	Clastogenic at toxic doses only	Sheldon et al. (1985a)
Micronucleus formation	Human peripheral blood lymphocyte culture	1–4000 µg/ml	99%	Negative	Ribas et al. (1997/8)
Micronucleus formation, optimized to detect excision repair	Human peripheral blood lymphocyte culture	25–100 µg/ml	99%	Negative	Surrallés et al. (1995)
Unscheduled DNA synthesis	Human epithelial-like cells	20–2000 µg/plate	NS	Positive, without dose–response relationship	Benigni et al. (1979)
Unscheduled DNA synthesis	Rat thymocytes and human peripheral blood lymphocytes	Rat thymocytes: 180–1800 µg/ml, human lymphocytes: 900 µg/ml	95%	Equivocal	Rocchi et al. (1980)
Unscheduled DNA synthesis	Rat primary hepatocytes	10 ⁻⁹ –10 ⁻² mol/l	Paraquat dichloride 99.6%	Negative	Trueman et al. (1985)
Comet assay for DNA damage	Human peripheral blood lymphocytes	≤2000 µg/ml for 4 h	99%	Positive (+S9) Positive (–S9)	Ribas et al. (1995)

Table 9. Continued

End-point	Test-object	Concentration/Dose	Purity	Results	Reference
Comet assay for DNA damage	Rat alveolar macrophages and epithelial type II cells	0–10 µmol/l	NS	Positive	Dušinská et al. (1998)
Comet assay for DNA damage	Chinese hamster cells	1–5 mmol/l	NS	Negative	Speit et al. (1998)
Comet assay for DNA damage	Rat astroglial cells	20–80 µmol/l	NS	Positive	Frederiksen & Clausen (1999)
Comet assay for DNA damage	Human cell line A549 and THP-1	10–100 µmol/l	NS	Positive	Don Porto Carera et al. (2001)
Comet assay for DNA damage	Human cell lines HeLa and Hep G2 and human peripheral lymphocytes	0–350 µmol/l	NS	Positive	Petrovská & Dušinská (1999)
Chromosomal damage	Chinese hamster fibroblasts	0.2–0.8 mg/ml 3 h	NS	Positive	Sofuni & Ishidate (1988)
Chromosomal damage	Chinese hamster cells	0.8 mg/ml 3 h	NS	Positive	Sofuni et al. (1988)
Chromosomal damage	Chinese hamster V79 cells	1–5 mmol/l	NS	Negative	Speit et al. (1998)
Chromosomal damage	Mouse (male and female BALB/C) bone-marrow and germ cells	Bone marrow: 7–23 mg/kg bw (single intraperitoneal dose) or 1.5, 3.0 and 5.0 mg/kg bw per day intraperitoneally for 10 days. Germ cells: 1.5, 3.0 and 5.0 mg/kg bw per day intraperitoneally for 5 days	NS	Equivocal (repeat doses); Negative (single dose)	Rios et al. (1995)
<i>In vivo</i>					
Micronucleus formation	Mouse (C57 Bl/6J/Alpk)	51.75, 82.8 mg of paraquat ion/kg bw (single dose by gavage)	Paraquat dichloride, 33.07% w/w paraquat ion	Negative	Sheldon et al. (1985b)
Micronucleus formation	Mouse (male Swiss albino)	83 mg/kg bw per os	NS	Positive	Prabakaran & Moorthy (1998)
Micronucleus formation	Mouse (male ICR)	2 × 15 mg/kg bw intraperitoneally	98%	Positive	Melchiorri et al. (1998)
Micronucleus formation	Mouse (pregnant female Swiss)	10 or 20 mg/kg bw subcutaneously	99%	Negative	Pena et al. (1999)
Micronucleus formation	Mouse (male Swiss)	2 × 20 mg/kg bw ip	NS	Positive ^a	Ortiz et al. (2000)
Cytogenetics	Rat (outbred Wistar-derived)	6.5–19.0 mg/kg bw, daily for 5 days (as paraquat ion, by gavage)	Paraquat dichloride, 100%	Negative (fuzzy banded cells were seen) ^b	Anderson et al. (1978)
Cytogenetics	Rat (Alpk:AP Wistar-derived)	15–150 mg/kg bw single dose by gavage	Paraquat dichloride, 33.07% w/w paraquat ion	Negative	Howard et al. (1987)
Chromosomal damage	Mouse (male CFLD)	Single dose 60 mg/kg bw per os; 2.4 mg/kg bw per os twice per week for 6 weeks; single dose 15 mg/kg bw intraperitoneally; 0.55–5.5 mg/kg bw × 5 intraperitoneally	25% paraquat ion	Negative	Selypes et al. (1978)

Table 9. Continued

End-point	Test-object	Concentration/Dose	Purity	Results	Reference
Unscheduled DNA synthesis	Rat (Alpk: AP Wistar-derived)	45–120 mg/kg bw single dose by gavage	Paraquat dichloride (technical) 33.07% paraquat ion	Negative	Trueman & Barber (1987)
DNA damage	Rat (male Wistar)	20 mg/kg bw intraperitoneally	NS	Negative	Sørensen & Loft (1999)
Dominant lethal mutation	Mouse (male Swiss-Webster)	66 mmol/kg bw per day	NS	Negative	Pasi et al. (1974)
Dominant lethal mutation	Mouse (male CD-1)	0.04–4 mg ion/kg bw per day	23.8% paraquat ion	Negative	Anderson et al. (1976)

In three tests for micronucleus formation in vivo (two using intraperitoneal administration and one using administration per os), paraquat gave positive results. In these three tests, the doses used were high; it is thus possible to conclude that paraquat may induce chromosome damage at high doses in assays in bone marrow in vivo.

The hypothesis that these effects are caused by the well-established ability of paraquat to generate reactive oxygen species, which are not detoxified at high doses owing to saturation of cellular defensive mechanisms, is likely to be the explanation for the results discussed above. For such an effect it is likely there would be a threshold as, except at high doses, reactive oxygen species are rapidly detoxified.

A mechanistic study was carried out into the ability of paraquat to produce “fuzzy-banded” chromosomes from rat bone-marrow cells (Anderson et al., 1979). From this study, it was concluded that paraquat was interfering with staining performed by the Giemsa method.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a three-generation study of reproductive toxicity, Wistar-derived Alderley Park rats were given diets containing technical-grade paraquat (25.8% paraquat ion) at a concentration of 0, 30 or 100 mg/kg. These dietary concentrations were equivalent to intakes of 0, 2.0 and 6.67 mg of paraquat ion/kg bw per day. The F_0 animals started on the test diets when aged 35 days, and they and their progeny remained on the diet throughout the study. The animals were examined daily and body weight and food consumption were recorded weekly. For the first mating, one male and two females receiving paraquat at the same dietary concentration were housed together. This was done at 105 days and produced the F_{1a} generation. The litters were examined after parturition and the number of live-born and stillborn pups recorded, together with the clinical state of the former. Offspring were examined daily for the number of live or dead offspring, and at 21 days, each litter was counted, weighed, sexed and autopsied. After 10 days, the second mating was carried out as described above. From the F_{1b} pups produced, 12 male and 24 female weanlings were selected to become the F_1 parents. The remainder of the offspring were killed and examined. At 100 days, one male and two females receiving paraquat at the same dietary concentration were housed together.

This mating produced the F_{2a} generation. The litters were examined after parturition and the number of live-born and stillborn pups was recorded, together with the clinical state of the former. Offspring were examined daily for the number of live or dead offspring, and at 21 days each litter was counted, weighed, sexed and autopsied. After 10 days, the second mating was carried out as described above. From these F_{2b} pups, 12 male and 24 female weanlings were selected to be the F_2 parents. Again two litters were produced (F_{3a} and F_{3b}), but this time all the offspring were killed and examined at 5–7 weeks. Tissues from 10 progeny of each sex per dietary concentration were examined histopathologically. No test material-related clinical effects were seen. Increased body-weight gain was seen in the groups of male rats receiving paraquat. This was particularly marked in the F_0 rats and was noted from 6 weeks onwards at both 30 and 100 mg/kg; it also occurred in the F_{1a} and F_{1b} rats, for which the body weights of females were also increased; this finding was not noted in the F_2 rats. Paraquat had no significant effect on food consumption. No adverse treatment-related effects were seen on reproductive performance (number of pregnancies to term, mean litter size, pup sex distribution and body weight at weaning). In the F_{1b} litters, the mean litter size was smaller at 30 mg/kg than in the controls or at 100 mg/kg. In the F_2 generation, litter size was increased at 100 mg/kg, but was within the range for historical controls. On histopathological examination, hydropic change was found in the renal tubules of weanlings that had been fed paraquat at a dietary concentration of 100 mg/kg. The NOAEL for the offspring was therefore 30 mg/kg (equivalent to 2.0 mg of paraquat ion/kg bw per day) on the basis of renal tubular changes in the weanlings at the highest dietary concentration. The NOAEL for the parents and for reproductive toxicity was 100 mg/kg (equivalent to 6.67 mg of paraquat ion/kg bw per day), the highest dietary concentration administered (Fletcher et al., 1972).

In another multigeneration study, Wistar-derived Alderley Park rats were fed diets containing technical-grade paraquat dichloride (32.7% w/w paraquat ion) at a concentration of 0, 25, 75 or 150 mg/kg as paraquat ion. These diets provided intakes equivalent to 0, 1.67, 5.0 and 10 mg of paraquat ion/kg bw per day. The F_0 parents comprised 15 male and 30 female rats per group; these rats were mated 12 weeks after the start of the study to produce the F_{1a} litters and 7 days after the last F_{1a} litter had been weaned (at 21 days) the F_0 parents were remated to produce the F_{1b} litters. The F_{1b} litters were weaned at 28 days. F_1 parents (30 females and 15 males per group) were selected from the F_{1b} litters and mated 11 weeks later to produce the F_{2a} and 7 days after the last F_{2a} litter had been weaned (at 28 days), remated to produce the F_{2b} litters (these were also weaned at 28 days). The F_2 parents were selected from the F_{2b} litters and mated 11 weeks later. All male parents were killed after mating to produce the F_{1b} or F_{2b} litters and the females were also killed, but after weaning of the F_{1b} or F_{2b} litters. The F_0 and F_2 parents were subjected to gross examination post mortem and the testes of all the males were fixed and processed for histopathological examination. Lungs from eight males and eight females of each group, and any abnormal tissues from all the animals were also fixed and processed for histopathological examination. Of the F_1 parents, 25 females and 10 males per group were subjected to a full autopsy and a wide range of tissues were processed for histopathological examination. Litters were examined at least daily and dead or abnormal pups removed for examination. Live and stillborn pups were counted and sexed at 24 h and 4, 10 and 21 days post partum. Pup weights were measured at 24 h and at 4, 10, 21 and 28 days post partum. All grossly abnormal pups and those found dead up to 18 days post partum were taken for teratological examination. Those aged > 18 days were taken for histopathological examination. Of the pups of the F_{1a} , F_{2a} and F_{3a} litters, about 50% were discarded, the remainder being subjected to gross necropsy; any abnormal tissues were processed for histopathological examination. After selection of

the parents for the next generation, five males and five females from the F_{1b} and F_{2b} litters and 10 males and 10 females from the F_{3b} litters were subjected to detailed histopathological examination. Test diets were fed throughout the study. The rats were observed daily, with a more detailed observation once per week, clinical observations and mortality were recorded. Body weights and food consumption were recorded weekly throughout the pre-mating period. During the pre-mating period, urine was taken from three males and three females per group for analysis for paraquat. After the pre-mating period, the male rats were weighed at 4-weekly intervals. No adverse effects were noted on parental clinical status, body weights or food consumption. Mortality was seen in female F_0 and F_1 parents receiving paraquat at the highest dietary concentration, mostly during or just after suckling a litter, no such effect being seen with the F_2 parents. No effect on body weight attributable to the test material was seen in the parents. There was some indication of an increase in food consumption in the F_0 parents and a decrease in food consumption in the F_1 parents. As these effects were not clearly dose-related, it is difficult to attribute them to paraquat: moreover, no effect on food consumption was noted with the F_2 parents. Measurements of urinary paraquat showed that dose-related absorption of paraquat occurred during the study. There were no adverse effects on fertility of the F_0 parents, male or female, during production of either F_1 litter. At 25 mg/kg (F_{1b}) and 75 mg/kg (F_{1b}), there was a reduction in the duration of gestation; in view of the lack of any such finding at higher dietary concentrations, this is unlikely to be related to the diet. There were no treatment-related effects on live-born offspring, maternal neglect or survival indices. In production of the F_2 litters by the F_1 parents, no effect of the paraquat was seen on fertility, body-weight gain of the pregnant dams, duration of gestation or live-born offspring, survival indices or litter size. No adverse effects on male or female fertility were noted in the F_2 parents on male or female fertility during production of the F_3 litters. Body-weight gain of the F_2 females when pregnant with F_{3a} or F_{3b} litters was increased at 75 mg/kg. The offspring of all three generations were healthy during lactation, although mortality in the F_{1b} litters was higher than that in the other litters. There were some differences in F_1 and F_3 litter weights between the groups, but they were not dose-related. Three of the F_0 female parents receiving paraquat at the highest dietary concentration died, and the lungs of these animals showed alveolar oedema, perivascular oedema and inflammatory cell infiltration (mainly macrophages, with a few neutrophils); profibroblasts and early fibrosis was also observed. Four lactating females receiving paraquat at the highest dietary concentration and suckling the F_{1b} litters died or were killed in extremis and their lungs showed similar changes. At termination, significant histopathological changes were confined to animals receiving paraquat at 150 mg/kg. These changes comprised consolidation, with alveolar fibrosis, epithelialization and infiltration with a few macrophages and profibroblasts. There was also hypertrophy and hyperplasia of the bronchial epithelium, with perivascular oedema and mixed inflammatory cell infiltration. No other treatment-related findings were seen in the F_0 female rats. In the F_0 male rats, there was an increase in focal histiocytosis at 75 and 150 mg/kg. In the F_1 parents, 13 females dying during late lactation were from the groups receiving 150 mg/kg and had lung changes similar to those described above. At termination of the F_1 females, five of the 17 surviving rats at 150 mg/kg had mild to severe lung changes. There was an increase in focal alveolar histiocytosis in the lungs at 75 and 150 mg/kg. This change was also present in the lungs of the male survivors at termination at 75 and 150 mg/kg. In the F_2 parental females, six rats at 150 mg/kg, which died or were killed in extremis, lung changes were observed at histopathological examination. At termination of the F_2 parental survivors, a proportion of both males and females at 150 mg/kg had some lung changes, as described above, and there was an increase in focal alveolar histiocytosis in the lungs at 75 and 150 mg/kg. Histopathological changes in the reproductive system were not seen in the parental animals of either

sex. In the offspring, mild perivascular inflammatory cell infiltration was seen in lungs of four out of five male and two out of six female F_{1b} offspring at the highest dietary concentration. Otherwise, there were no pathological changes in the F_1 , F_2 or F_3 offspring that could be directly attributed to the test material, although one F_{3b} litter starved (and died or were sacrificed in extremis) as a result as a result of the death of the mother. The NOAEL was 25 mg/kg, equivalent to 1.67 mg of paraquat ion/kg bw per day, on the basis of lung changes at 75 and 150 mg/kg in adult rats. The NOAEL for reproductive toxicity was 150 mg/kg, the highest dietary concentration administered, this being equivalent to 10 mg of paraquat ion/kg bw per day. The NOAEL for toxicity in the offspring was 75 mg/kg, equivalent to 5.0 mg of paraquat ion/kg bw per day (Lindsey et al., 1982).

In another three-generation study, groups of 30 male and 30 female Sprague-Dawley (CRJ:CD) rats were fed diets containing paraquat dichloride (purity, 98.6%) at a concentration of 0, 100, 200 or 400 mg/kg. The intakes of test material achieved are given in Table 10.

The F_0 parents received the diets from week 5 weeks until after weaning of the second (F_{1b}) litters. There was a 13-week pre-mating period after which the males and females were mated to produce the F_{1a} litters. The pups were examined for number of live born, stillbirths, sex ratio and external abnormalities. Eight pups per litter were investigated until weaning at 21 days after birth, and the pups were then examined post mortem. Two weeks after the F_{1a} litters had been weaned, the F_0 females that were successful breeders were housed with their previous mates to produce the F_{1b} litters. The F_0 females bearing the F_{1b} litters were divided into three groups, five females being used for teratology, five for post-natal investigations and 10 to produce the F_1 parents as follows. Five pregnant females of each group were killed on day 20 of gestation. After macroscopic examination, the uteruses were removed and examinations carried out to determine number of live fetuses, fetal deaths and resorptions. Live fetuses were weighed, sexed and examined for external abnormalities. One-third of all live fetuses were fixed in Bouin solution, examined in detail and dissected. The remainder were fixed in 95% alcohol, stained with alizarin red S and examined for skeletal abnormalities. Five pregnant females underwent parturition and the duration of gestation and numbers of live pups and stillbirths were recorded, together with sex ratio and any external abnormalities of the pups. Where there were more than eight pups per litter, the excess were stained with alizarin red S and examined for skeletal abnormalities. The remaining live pups were retained until weaning at 28 days, when they underwent exami-

Table 10. Measured intake of paraquat dichloride (mg/kg bw per day) in a three-generation study

Group	Dietary concentration (mg/kg)			
	0	100	200	400
F_0 males	0	6.6	13.0	25.1
F_0 females	0	7.2	13.8	29.3
F_{1b} males	0	9.6	19.8	38.7
F_{1b} females	0	10.2	20.8	32.9
F_{2b} males	0	8.5	16.9	40.9
F_{2b} females	0	9.6	19.6	48.7
Mean for males*	0 (0)	8.2 (5.9)	16.6 (12.0)	34.9 (25.3)
Mean for females*	0 (0)	9.0 (6.5)	18.1 (13.1)	37.0 (26.8)

From Suzuki et al. (1983)

* Intake of paraquat ion in parentheses

nation post mortem. Ten pregnant females underwent parturition and the duration of gestation and numbers of live pups and stillbirths were recorded, together with sex ratio and any external abnormalities of the pups. Where there were more than eight pups per litter, 4 days after birth, the excess were killed, stained with alizarin red S and examined for skeletal abnormalities. The remaining live pups were retained until weaning at 21 days. Mortality, viability and growth status were recorded, and 30 males and 30 females at each dietary concentration were selected to be the next generation, the remainder being autopsied with their (F_0) dams. The F_1 rats, which produced the F_{2a} and F_{2b} litters, were treated similarly to the F_0 rats (see above), being fed the diets from the time they were weaned until the weaning of their second (F_{2b}) litters. Ten pregnant females were, however, used for teratology studies and 10 for postnatal investigation, any remaining being allowed to give birth, after which the dams and pups were sacrificed. The third generation (from the F_{2b} litters) were fed the diets from the time they were weaned until at least 13 weeks later.

Throughout the study, all animals were examined daily and the F_0 and F_{1b} females were weighed on days 0, 7, 14 and 20 day of gestation and on days 0, 7, 14 and 21 post partum (during lactation). The F_{1b} litters to be used for postnatal investigation were weighed 0, 4, 7, 14, 21 and 28 days after birth, and those that were used to produce the next generation were weighed 0, 4, 7, 14 and 21 days after birth. Food consumption was measured weekly for each cage, but not during dosing of the F_0 generation and not in the mating period that produced the F_{1b} litters. Water consumption by 10 males and 10 females per dietary concentration was measured weekly, except for the F_0 mating period and the mating period that produced the F_{1b} litters. At autopsy of the parental rats, selected organs were weighed, and these and other selected organs were fixed and processed for histopathological examination.

No deaths were seen in the F_0 parents, but excess deaths were seen in the subsequent generations. At 400 mg/kg, five F_{1b} females died (compared with two of the controls). There were 14 deaths or animals killed in extremis in F_{2b} males at 400 mg/kg, and 10 deaths or rats killed in extremis in F_{2b} females at 400 mg/kg. At 400 mg/kg, wheezing was heard in rats of each generation and this was often accompanied by weight loss. At 400 mg/kg, there was a reduction in body-weight gain in male and female F_0 and F_{2b} rats at an early stage during dosing, and in female F_{1b} rats during gestation and lactation. There was a reduction in food consumption in F_0 and F_{2b} rats at 400 mg/kg early in the dosing period. No treatment-related changes were seen in reproductive parameters (corpora lutea, implantation number, implantation (%), number of dead and live fetuses, sex ratio or placental weight), nor were any teratogenic effects seen. Retarded ossification was noted in fetuses from the F_0 dams at 100 mg/kg, and in those from the F_{1b} dams at 100 mg/kg and 400 mg/kg. Furthermore, there were reductions in body weights of male fetuses from the F_{1b} females at 100 and 400 mg/kg. Retardation of ossification was also noted in fetuses from all test groups of F_{1b} dams. There were reductions in body-weight gain in F_{2b} pups at 400 mg/kg. There was retarded opening of the vagina in both the F_{1b} and F_{2b} female pups at 400 mg/kg. No effects on organ weights were seen that were clearly attributable to the test material. However, F_0 animals at 400 mg/kg showed a reduction in brain weight, both absolute and relative. Histopathologically, alveolar hyperplasia and fibrosis was found in F_0 males at 400 mg/kg, in F_{1b} females at 100, 200 and 400 mg/kg, and in F_{2b} rats of both sexes at 400 mg/kg. At 400 mg/kg, F_{1b} rats also showed atelectasis, congestion and haemorrhage, while in the decedents from F_{2b} rats at 400 mg/kg, alveolar wall hyperplasia and fibrosis, atelectasis, congestion, haemorrhage and oedema were found. The LOAEL for maternal toxicity was 100 mg/kg on the basis of lung changes seen in female F_{1b} rats (this dietary

concentration is equal to 9.0 mg of paraquat dichloride/kg bw per day and 6.5 mg of paraquat ion/kg bw per day). No NOAEL for fetal toxicity was seen because of retarded ossification and decreased body weight, the LOAEL for fetal toxicity being 100 mg/kg (equal to 9.0 mg of paraquat dichloride/kg bw per day and 6.5 mg of paraquat ion/kg bw per day in the dams.² The NOAEL for pup toxicity was 200 mg/kg on the basis of decreased body weight in F_{2b} pups at 400 mg/kg and retarded opening of the vagina in F_{1b} and F_{2b} female pups at 400 mg/kg. This NOAEL is equal to 16.6 mg of paraquat dichloride/kg bw per day in males and 18.1 mg of paraquat dichloride/kg bw per day in females (12.0 and 13.1 mg of paraquat ion/kg bw per day in males and females, respectively). The NOAEL for reproductive toxicity was 400 mg/kg, (the highest dietary concentration). This is equal to 34.9 mg of paraquat dichloride/kg bw per day and 25.3 mg of paraquat ion/kg bw per day in males, and 37.0 mg of paraquat dichloride/kg bw per day and 26.8 mg of paraquat ion/kg bw per day in females. An overall NOAEL for the study was not elicited, as histopathological evidence of lung damage was found at all dietary concentrations in F_{1b} female rats, and delayed ossification and reductions in body weight in fetuses were seen at ≤ 100 mg/kg. The overall LOAEL for the study was 6.5 mg of paraquat ion/kg bw per day (Suzuki et al., 1983).

Mice

In a two-generation study of reproductive toxicity, groups of 24 pairs of ICR albino mice (paired at age 30 days) and given diets containing paraquat at a concentration of 0, 45, 90, or 125 mg/kg (equal to 0, 45, 90 or 125 mg of paraquat ion/kg feed and equivalent to 0, 6.75, 13.5 or 18.75 mg of paraquat ion/kg bw per day). Females were allowed 8 weeks from pairing to produce a litter and cages were checked daily for parental and pup mortality. The pups were weaned after 30 days and either segregated or paired for use in producing the second generation. Exposure of the parental (F₀) mice continued until the weaning of the F₁ mice, which were exposed to the diet for 49 days postnatally. Lungs were excised from sucklings, weanlings and adults in groups in which mortality had been observed, and were processed for histopathological examination. At age 30 days, randomly selected F₁ mice were paired (not siblings) to produce the next generation. The control group comprised 24 pairs and the groups receiving paraquat comprised two groups of 12 pairs at each dietary concentration. One group of 12 pairs at each dietary concentration was removed from the test diet and placed on control diet on weaning, whereas the other remained on the same diet as their parents. No differences were observed in the age of females at first parturition, pups borne/litter or in pup abnormalities; at the highest dietary concentration, however, the number of pairs of mice producing litters was reduced because of maternal deaths. Furthermore, effects on the mortality of F₁ offspring were observed at the highest dietary concentration. The age of F₁ females at second parturition was increased, and mortality in the F₂ generation at 7 weeks was increased at 125 mg/kg. Excess mortality was not observed in the F₁ parents. Maternal and offspring lungs were histopathologically abnormal, with extensive fibrosis at the highest dietary concentration and in a few instances, in the dams, at the intermediate concentration. The NOAEL for the study was 45 mg/kg, equivalent to 6.75 mg of paraquat ion/kg bw per day. The NOAEL for pup toxicity was 90 mg/kg (equivalent to 13.5 mg of paraquat ion/kg bw per day) on the basis of excess mortality and histopathological changes in the lungs. Specific reproductive toxicity was not seen (Dial & Dial, 1987).

² Assuming no paternal effect.

(b) *Developmental toxicity**Rats*

In a study of developmental toxicity, groups of 29 or 30 rats (strain not stated) were given paraquat dichloride (purity, 100%) at a dose of 0, 1, 5, or 10 mg of paraquat ion/kg bw per day by oral gavage on days 6–15 of gestation. Animals were examined daily and maternal body weight was measured on days 0, 3, 6, 8, 12, 16 and 21. Food consumption was not measured. On day 21 of gestation the animals were killed and their uteri were examined for live fetuses and resorptions; corpora lutea were counted. Fetuses were removed, weighed, sexed and observed for gross malformations, then preserved before examination for soft tissue or skeletal abnormalities. Alternate fetuses were examined for soft tissue or skeletal abnormalities. Maternal lungs and kidneys from at least 11 surviving rats per group were fixed and processed for histopathological examination. Observed mortality in the group receiving the highest dose was attributed to paraquat. Clinical signs of maternal toxicity occurred in many animals at 5 mg/kg bw per day and in most animals at 10 mg/kg bw per day. These signs were piloerection, weight loss, hunched appearance and, sometimes, respiratory distress. Reduced maternal body-weight gain was seen at 5 and 10 mg/kg bw per day, the effect being greater at the higher dose. The decedent dams at the highest dose showed, grossly, patchy red areas in the lungs, while microscopically there was alveolar oedema with polymorphonuclear infiltration. Proximal tubular degeneration in the kidneys was also found. These changes were not present in the groups receiving a dose of 5 mg/kg bw per day or the survivors to 21 days in any group. Slightly reduced mean fetal weights were seen at 5 and 10 mg/kg bw per day (the significance at $p < 0.05$ at 5 mg/kg bw per day depended on one female who had 12 resorptions out of 14 implants, and the two fetuses were very small). Significant intergroup differences in fetal survival, number of viable fetuses, proportion of females with resorptions, numbers of corpora lutea and sex ratios were not seen. If, however, the female receiving a dose of 5 mg/kg bw per day that had 12 resorptions out of 14 implants, and whose two fetuses were very small was included, a difference was apparent in viable fetuses as a proportion of implant numbers between the control group and the group receiving a dose of 5 mg/kg bw per day. No intergroup differences in skeletal abnormalities were found, but retarded ossification (caudal vertebrae and forelimb and hindlimb digits) was seen at 5 and 10 mg/kg bw per day. No fetal soft-limb abnormality was found that was attributable to treatment. The NOAEL for maternal and fetal toxicity was 1 mg/kg bw per day on the basis of clinical signs, and reduced body-weight gain in the dams and reduced mean fetal weights and retarded ossification in the fetuses. Teratogenicity was not observed (Hodge et al., 1978a).

In a study of developmental toxicity, groups of 24 female Alpk:ApfSD Wistar-derived rats were given technical-grade paraquat dichloride (paraquat ion, 38.2% w/v) at a dose of 0, 1, 3 or 8 mg of paraquat ion/kg bw per day by gavage on days 7–16 of gestation. Clinical observations were recorded daily and body weight was recorded on days 1, 4, 7–16, 19 and 22 of gestation. Food consumption was recorded over 3-day periods: days 1–4, 4–7, 7–10, 10–13, 13–16, 16–19 and 19–22. On day 22 of gestation, the rats were killed and their uteri weighed and examined for live fetuses and intrauterine deaths. The fetuses were weighed, examined for external and visceral abnormalities, sexed, eviscerated and stained for skeletal examination. No compound-related adverse clinical finding was recorded. There was a small amount of weight loss at the highest dose between days 1 and 2 of dosing (days 7–8 of gestation) and the difference in weight between the group receiving the highest dose and the controls was significant on days 8–14 and 16 of gestation. The effect on body weight of the dams at the highest dose (8 mg of paraquat ion/kg bw per day) was considered to be

test material-related. Developmental toxicity was not seen. Paraquat was not teratogenic. The NOAEL for maternal toxicity was 3 mg of paraquat ion/kg bw per day on the basis of effects on body weight at 8 mg of paraquat ion/kg bw per day, and the NOAEL for developmental toxicity was 8 mg of paraquat ion/kg bw per day, the highest dose tested (Hodge, 1992).

Mice

In a study of developmental toxicity, groups of 26–37 pregnant mice were given paraquat dichloride (purity, stated to be 100%) at a dose of 0, 1, 5 or 10 mg of paraquat ion/kg bw per day by gavage on days 6–15 of gestation. The animals were observed daily and weighed on days 0, 3, 6, 9, 12, 15 and 18 of gestation. Food consumption was not measured. On day 18, the mice were killed and their uteri were examined for resorptions. Fetuses were removed, weighed, sexed and observed for gross abnormalities, and preserved for examination for soft tissue or skeletal changes. In the mothers, for at least eight animals per group, lungs and kidneys were fixed and processed for histopathological examination. No adverse clinical signs were noted. Maternal body-weight gain was decreased during gestation at 5 and 10 mg/kg bw per day, but only at 5 mg/kg bw per day was the difference from that of controls significant. There were no test material-related effects on maternal pathology. The numbers of implantations, viable fetuses and resorptions, sex ratio and fetal and litter weights were not different between treated and control groups. There was a higher incidence of fetal umbilical hernia at 5 mg of paraquat ion/kg bw per day, but this was considered to be unrelated to dosing. There was no increase in skeletal or soft tissue abnormalities and ossification was not retarded. The NOAEL for maternal toxicity was therefore 10 mg of paraquat ion/kg bw per day, the highest dose tested (since the effects based on reduced weight gain in pregnancy were not dose-related), while the NOAEL for fetal toxicity was also 10 mg of paraquat ion/kg bw per day, the highest dose tested (Hodge et al., 1978b).

In a study of developmental toxicity, groups of 26 Crl:CD1 (ICR) BR mice were given using technical-grade paraquat dichloride (purity, 38.2%) at a dose of 0, 7.5, 15 and 25 mg of paraquat ion/kg bw per day by gavage on days 6–15 of gestation. Maternal mortality (mice that died or were killed in extremis) and clinical signs were recorded daily from the start of gestation. Body weights were recorded on days 0, 6–15 and 18 of gestation. Food consumption was recorded over days 0–6, 6–9, 12–15 and 15–18 of gestation. The remaining females were killed on day 18 of gestation. Females were examined post mortem, when the lungs (with the trachea) and kidneys were weighed. Gestation status was assessed and the gravid uterine weight was recorded. The number of live and dead implantations was recorded. Live fetuses were weighed, examined for external abnormalities and sexed. One-half of the fetuses were examined for visceral abnormalities, and then for skeletal abnormalities, the other half being examined for visceral abnormalities. At the highest dose, there were five decedents at 15–17 days (four killed in extremis and one found dead). In the four killed in extremis, piloerection, laboured breathing, hunched posture, hypothermia, hypoactivity and pallor of the extremities and eyes were observed. No other treatment-related clinical effects were observed. Also at the highest dose, there was a decrease in body-weight gain over days 12–15 and 15–18, and over the whole period of dosing (days 6–15 of gestation); furthermore, body weight in the group receiving the highest dose was lower than that in the controls on day 15 and day 18 of gestation. Body weight and weight gain were unaffected at the lower doses. Significant differences in food consumption were seen on analysis of variance. Although food consumption between days 12 and 15 was reduced in the group receiving the highest dose compared with that in the controls, the difference was

not significant. Food consumption was not reduced in mice at the lower dose. Despite the lack of statistical significance, the present reviewer considered that the reduction in food consumption between days 12 and 15 in the group receiving the highest dose compared with that in the controls may be biologically significant. At necropsy of the mice killed in extremis, dark red patches were found in the lungs. In all mice, absolute and relative lung weights were increased at the highest dose. The difference in absolute but not relative lung weights between the groups disappeared, if the decedents were excluded. The number of implantations, live fetuses, postimplantation loss and fetal sex ratio were not affected by treatment. At the highest dose, retardation of fetal growth was seen and mean fetal weight was decreased. No treatment-related effect on the prevalence of major abnormalities was seen. At 7.5 mg/kg bw per day and 15 mg/kg bw per day, but not 25 mg/kg bw per day, there were more fetuses and litters with minor external/visceral abnormalities, but as this did not appear to be dose-related, the effect was not considered to be treatment-related; this effect was due to an increase in the number of fetuses with renal pelvic cavitation. At the highest dose, there was retardation of ossification of the caudal vertebrae and the occipital and astragalus bone, with misshapen sternebrae. No treatment-related effect was seen at the lower doses. The NOAEL for maternal and fetal toxicity was 15 mg of paraquat ion/kg bw per day on the basis of effects on body weight, reduced food consumption and lung changes in the dams and retardation of ossification in the fetuses at the highest dose tested. Teratogenicity was not seen at any dose (Palmer, 1992).

Groups of Swiss-Webster mice were given paraquat at a dose of 1.67 or 3.35 mg/kg bw per day intraperitoneally or 20 mg/kg bw per day by gavage on days 6–16 of gestation. Gravid mice were sacrificed on day 19 of gestation. The number of live and dead fetuses and resorptions was recorded and the fetuses were removed, dried and examined for gross defects. Equal numbers of pups from each litter were fixed for examination of soft tissue or skeletal anomalies. No teratogenic effect was observed, although a slight degree of non-ossification of sternebrae was seen at all doses. Fetotoxicity, as evidenced by increased resorption (%), was seen at only 3.35 mg/kg bw per day intraperitoneally. At no dose was the number of fetuses, or their mean body weight affected by treatment. The amount of radiolabel reaching the mouse embryo when ^{14}C -labelled paraquat at a dose of 3.35 mg/kg bw administered intraperitoneally or 20 mg/kg bw administered orally on day 11 of gestation was small (Bus et al., 1975).

The developmental toxicity of paraquat was determined in Sprague-Dawley rats treated intravenously with paraquat at a single dose of 15 mg/kg bw on a single day, one of days 7–21 of gestation. The number of live and dead fetuses and resorptions was counted at day 22 (or before for decedent dams). Excess maternal deaths occurred with paraquat compared with controls receiving saline only, and there was an increase in the number of dead and resorbed fetuses (Bus et al., 1975).

Groups of pregnant Swiss-Webster mice were given drinking-water containing paraquat (purity unstated) at a concentration of 50 or 100 mg/l (and 150 mg/l) from day 8 of gestation until postnatal day 42. Pregnant mice receiving paraquat at 150 mg/l died during gestation (at about day 16). Treatment with paraquat at 100 mg/l and 50 mg/l did not alter the postnatal growth rate, nor was postnatal mortality increased at 50 mg/l. Administration of drinking-water containing paraquat at 100 mg/l caused an increase in postnatal mortality, and an increase in the sensitivity of pups to oxygen toxicity on postnatal days 1 and 28, while drinking-water containing paraquat at 50 mg/l did not. At both 50 and 100 mg/l, the sensitivity to oxygen toxicity and to bromobenzene at postnatal day 42 was increased. The

authors considered that the effect of bromobenzene could be caused by depletion of reduced glutathione (Bus & Gibson, 1975).

2.6 *Special studies*

(a) *Mechanistic studies*

(i) *Histopathological studies on the lung*

Small groups of A/He mice were given drinking-water containing paraquat at a concentration of 50–300 mg/l, and retained for 1 to 16 weeks (further details of the material used are not given in the paper). Detailed light and electron microscopical studies were carried out on the mice post mortem. The main findings on light microscopy were vascular dilatation and veins filled with platelets and erythrocyte aggregates. At the higher doses, interveolar septal thickening was seen. At ≥ 100 mg/l, focal or, sometimes, lobar pneumonitis was observed, with small mononuclear cells, macrophages and neutrophils. In those mice receiving paraquat for 4 weeks or more, fibroblasts were seen in the septal walls. Obliteration of air spaces was seen. Type II cells were observed to be undamaged on electron microscopy in this study, but type I cells were swollen and there was evidence of oedema of interalveolar septa. The alveolar air spaces were filled with a clear exudate and where there was consolidation, fibroblasts and collagen were observed. Lymphocytes and plasma cells were noted (Brooks, 1971).

In a study of the ultrastructure of the rat lung after administration of paraquat, 51 female Wistar albino rats were divided into 17 groups, each group comprising two test animals and one control. On day 1, animals in 15 groups received paraquat at a dose of 40 mg of paraquat ion/kg bw administered intraperitoneally, while groups 16 and 17 received paraquat at a dose of 30 mg of paraquat ion/kg bw. At intervals of between 10 min and 4 h after injection, the animals were killed and the left lungs were fixed with glutaraldehyde via the main bronchus, and processed for electron microscopy. The right lungs were processed for light microscopy. Using light microscopy, changes were not seen until 24 h. After 2 days, microscopy revealed interstitial oedema and a fibrinous exudate, with a polymorph infiltration, which was more widespread after 4 days. Pro-fibroblasts were seen in the vicinity of bronchioles and major blood vessels. Using electron microscopy, after 4 days there was an increase in the quantity of rough endoplasmic reticulum and numbers of mitochondria and free ribosomes in alveolar type I cells. The cells were also thicker. These changes were followed by swelling of mitochondria, fragmentation of the rough endoplasmic reticulum and a reduction in cellular density. Later the cells disintegrated. Changes in the type II alveolar cells did not occur until 8 h and were not pronounced until 18 h after administration of paraquat. The changes consisted of swelling and rupture of the mitochondria, fusion and vacuolation of lamellar bodies and disruption of the cytoplasm. Three days after administration of paraquat, pro-fibroblasts were seen in the alveolar spaces (Smith & Heath, 1974).

In other species, such as rats and dogs, histopathological appearances after treatment with paraquat are generally similar to those in mice (Clark et al., 1966), although Butler (1975) found that the Syrian hamster relatively resistant to interstitial fibrosis. Butler & Kleinerman (1971) found that the New Zealand white rabbit did not develop pulmonary changes typical of paraquat poisoning in other species, despite intraperitoneal administration of paraquat at a dose of 2–100 mg/kg bw and sacrifice of animals being delayed up to 1 month. The only findings in the lungs were occasional small interstitial infiltrates of lymphocytes and plasma cells, minimal alveolar hyperplasia and some alveolar macrophages.

(ii) *Mechanism of uptake by pneumocytes*

A considerable amount of work has been done on the mechanisms that underlie the toxicity of paraquat. The fact that paraquat is concentrated by the lungs has been discussed above. Rose et al. (1976) showed that lung slices from rats Wistar-derived Alderley Park rats, beagle dogs, New Zealand white rabbits and cynomolgus monkeys (*Macaca fascicularis*) could concentrate paraquat via the polyamine active uptake system. This is the system by which paraquat and the structurally similar polyamines, such as putrescine and spermidine, are accumulated by type II alveolar cells (see reviews by Smith, 1985, Smith et al., 1990 and Lock & Wilks, 2001).

The uptake kinetics of paraquat and putrescine and their mutual inhibition in freshly isolated rat type II cell suspensions was reported. The uptake of paraquat by type II cells exhibited saturation kinetics and could be inhibited in a concentration-dependent manner by putrescine. The authors postulated that the polyamine uptake pathway in type II cells for paraquat and putrescine possessed two separate sites, one for each substrate, and that binding at one site leads to a conformational change in the other (Chen et al., 1992).

(iii) *Production of cell damage in the lung*

A study in which drinking-water containing paraquat at a concentration of 50 or 100 mg/l was administered to Swiss-Webster mice has already been discussed (see section on developmental toxicity). Drinking-water containing paraquat at a concentration of 100 mg/l increased postnatal mortality, and increased pups' sensitivity to oxygen toxicity at 1 and 28 days after birth, while drinking-water containing paraquat at a concentration of at 50 mg/l did not. At both 50 and 100 mg/l, drinking-water containing paraquat increased the sensitivity to oxygen toxicity and to bromobenzene at 42 days after birth (Bus & Gibson, 1975).

In a study of the hypothesis that the pulmonary toxicity of paraquat is caused by cyclic reduction–oxidation, with generation of superoxide radicals and singlet oxygen, and the production of lipid peroxidation, mouse lung microsomes in vitro were found to catalyse the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-dependent reduction of paraquat. Incubation of paraquat with NADPH, NADPH-cytochrome reductase and purified microsomal lipid increased the production of malondialdehyde (MDA) production. Addition of superoxide dismutase or 1,3-diphenylisobenzofuran (a singlet oxygen trapper) inhibited paraquat-induced lipid peroxidation. Toxicity caused by paraquat (purity unstated) in mice (strain unstated) was decreased by phenobarbital and increased by selenium, vitamin E or reduced glutathione deficiency. The toxicity of paraquat was increased by exposure to 100% oxygen (Bus et al., 1976b).

In similar studies in rats and mice, Bus et al. (1976a) showed that pretreatment with phenobarbital increased the LD₅₀ for paraquat in Swiss-Webster mice, but only when administration of the phenobarbital was continued after the administration of paraquat. Paraquat, administered intraperitoneally at a dose of 30 mg/kg bw, decreased liver concentrations of reduced glutathione and lung concentrations of lipid-soluble antioxidants. After receiving paraquat at a dose of 45 mg/kg bw, Sprague-Dawley rats habituated to 85% oxygen were found to have a longer median time to death than rats exposed to air. These rats were believed to have greater activity of lung enzymes that combat lipid peroxidation.

The effect of paraquat on oxidative radical reactions in the lung was evaluated by studying malondialdehyde production and chemiluminescence (spontaneous and induced

by tertiary butyl hydroperoxide) in the isolated rat lung. After 2 h of perfusion with paraquat at 3.0 mmol/l, malondialdehyde content in lung homogenates was 16 ± 7 nmol/g of dry weight higher than in control lungs; during 30 min of perfusion, malondialdehyde efflux was 33 ± 15 nmol/g of dry weight higher than in control perfusates. Spontaneous chemiluminescence was not increased by 2 h of perfusion with paraquat at concentrations ranging from 0.75 to 6.0 mmol/l. Chemiluminescence induced by tertiary butyl hydroperoxide, however, was $17 \pm 3\%$ higher immediately after the addition of hydroperoxide and reached a $16 \pm 6\%$ higher plateau for lungs perfused with paraquat than for control lungs. Spectral analysis of the light emitted during induced chemiluminescence demonstrated peak intensity between 630 and 730 nm for controls and for lungs treated with paraquat. Increased production of malondialdehyde and increased induced chemiluminescence indicated that perfusion with paraquat enhances lipid peroxidation in the isolated rat lung (Aldrich et al., 1983).

The redox cycling abilities of paraquat and nitrofurantoin, compared with those of the potent redox cyclers diquat and menadione, was studied in lung and liver microsomes using the oxidation of NADPH and consumption of oxygen. In terms of relative potencies of these compounds to undergo redox cycling, diquat and menadione were similar and much greater than paraquat, which was similar to nitrofurantoin. This was partly attributed to the much lower affinity (K_m) of lung and liver microsomes for paraquat and nitrofurantoin than for diquat and menadione. These data were considered to have important implications in assessing the risk of exposure to paraquat. Low concentrations of paraquat would not be expected to cause lung damage because insufficient compound would be present in the lung to exert toxicity by redox cycling (Adam et al., 1990).

There has been some disagreement over which cell type in the lungs is primarily affected by paraquat. Hirai et al. (1985) injected male Sprague-Dawley rats with paraquat dichloride at 40 mg/kg bw and observed mitochondrial swelling and loss of granules in alveolar type II cells at 6 h.

In a study of the effect on the lungs of paraquat applied to the skin over/next to the lungs of male Long-Evans rats, paraquat (as 1 ml of solution containing 8 g of paraquat) was applied weekly to the back of 18 rats. There were seven control rats. From week 4, two rats were killed per week. After 6 weeks, the concentration of the test solution was increased to 28.5 mg/ml. Lungs, kidneys, livers and the application site were removed at autopsy and processed for histopathological examination. In some of the rats receiving paraquat, there was evidence of intra-alveolar haemorrhage. The medial thickness of large and small pulmonary arteries in the test groups was greater than in the controls. No histopathological change was present in the livers and kidneys. There was necrosis and ulceration of the application site, with acute and chronic inflammatory cell infiltration (Levin et al., 1979).

(b) *Liver toxicity*

Liver toxicity, as revealed by elevated liver enzymes, jaundice, and histopathological changes in the liver at examination post mortem, is sometimes seen in cases of poisoning with paraquat in humans. A number of studies examining this phenomenon (e.g. Cagen & Gibson, 1977; Burk et al., 1980). Cagen & Gibson (1977) have found that, in Swiss-Webster mice, paraquat was not hepatotoxic, unless the mice were deficient in selenium.

(c) *Kidney toxicity*

In cases of poisoning in humans, renal tubular damage has been noted at autopsy. In a study of the nephrotoxicity of paraquat in vitro and in vivo, proximal tubular function was monitored in vitro by measuring the accumulation of *p*-aminohippurate and *N*-methylnicotinamide using renal cortical slices from Swiss-Webster mice poisoned with paraquat at the LD₅₀ for intraperitoneal administration (50 mg/kg bw). Tubular function in intact Swiss-Webster mice was estimated using disappearance of phenolsulthalein and [¹⁴C] paraquat from plasma in vivo. Glomerular function was estimated using disappearance of iothalamate from plasma in animals injected intravenously with paraquat at a dose of 50 mg/kg bw. Accumulation of *p*-aminohippurate and *N*-methylnicotinamide by renal cortical slices in vitro was not greatly altered. Disappearance in vivo of phenolsulthalein and [¹⁴C] paraquat from plasma was greatly reduced, but iothalamate disappearance was little affected. The authors concluded that nephrotoxicity attributable to paraquat affects primarily the proximal tubule (Ecker et al., 1975).

It has been noted that the uptake of paraquat by rat renal tubular cells in culture is saturable (Chan et al., 1996a). Of two renal tubular cell lines, one resembling proximal tubular cells and the other resembling distal tubular cells, the latter was found to be more resistant to the effects of paraquat (Chan et al., 1996b).

(d) *Neurotoxicology*

Paraquat is structurally similar to the known dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). As a result, paraquat has been considered as a possible etiological factor in Parkinson disease. Paraquat is, however, a dication and does not readily cross biological membranes and the blood–brain barrier, whereas MPTP readily crosses the blood–brain barrier and is oxidized to the dihydropyridinium ion and then the neurotoxic methylphenylpyridinium ion. The methylphenylpyridinium ion is taken up into dopaminergic neurones by the same uptake mechanism as dopamine itself (Fonnum, 1999). Moreover, it was reported that, in a study using an inducible system in neuroblastoma cells (described only in an abstract), the toxicity of paraquat was not mediated by the dopamine transporter (Miller & Quan, 2002). Furthermore, in another abstract it was reported that, while the methylphenylpyridinium ion inhibited dopamine re-uptake in rat and mouse synaptosomes, paraquat did not, and that paraquat had no binding affinity for the dopamine transporter and the D₁ and D₂ receptors (Foster et al., 2003). Shimizu et al. (2003) examined the mechanism by which paraquat is toxic to dopamine neurons in male Wistar rats in vivo, using GBR-12909, a selective inhibitor of the dopamine transporter. GBR-12909 reduced the uptake of paraquat into the striatal tissue, including dopaminergic terminals. Subcutaneous treatment with paraquat at 10 mg/kg bw for 5 days significantly decreased concentrations of brain dopamine and dopamine metabolites in the striatum. When paraquat was administered through a microdialysis probe stereotactically implanted into the striatum, a transitory increase in extracellular concentrations of glutamate, followed by long-lasting elevations of the extracellular concentrations of nitrite and nitrate and dopamine, were detected in the striatum of rats. This lasted for more than 24 h after treatment with paraquat and could be inhibited by *N*^G-nitro-L-arginine methyl ester, dizocilpine, 6,7-dinitroquinoxaline-2,3-dione and L-deprenyl.

The behavioural and neuropathological effects in rats of both systemic and intrahippocampal injections of paraquat dichloride were studied by Bagetta et al. (1992). Paraquat (0.1–1.0 μmol), injected into the dorsal hippocampus, produced seizures within a few

minutes of injection, and caused neuronal damage in the CA1 and CA3 pyramidal cell layers, pyriform cortex, dentate granule cell layer and in the hilus fascia dentata at 24 h ($n = 9$ rats). A smaller dose of paraquat (10 nmol) was ineffective. The effects of intrahippocampal injections of paraquat (1 μ mol) were prevented by coadministration with atropine (50 nmol). Systemic injections of paraquat (20–100 mg/kg bw) produced forelimb clonus and rearing in 10 out of 15 animals. Neuronal cell death was found 24 h later in nine of these rats and was restricted to the pyriform cortex, this being the region of the brain with the highest concentrations of paraquat. Atropine (at a dose of 150 mg/kg bw given intraperitoneally 60 min previously) completely prevented the motor seizures, but cell death still occurred in two of the six animals tested. The use of certain experimental treatments for intrahippocampal toxicity of paraquat has been studied by the same group of authors (Bagetta et al., 1994).

The effects of paraquat (1–5 μ g) on behaviour, morphology and neurochemistry were investigated in male Wistar rats treated by unilateral injection into the substantia nigra. There was vigorous contralateral rotational behaviour in response to administration of apomorphine. The animals were killed 2 weeks after dosing. Morphologically, there was loss of Nissl substance, glial reaction and loss of neurones in the substantia nigra, and neurochemically, there was dopamine depletion (Liou et al., 1996).

In a study of the behavioural and electrocortical effects of paraquat, Wistar-Morini rats were given paraquat administered by cannula into the substantia nigra, pars compacta, an area where dopamine-containing cell bodies are present, and into the caudate nucleus, where dopamine-containing nerve endings of the dopamine nigro-striatal system project. Paraquat was also administered into the locus coeruleus, an area containing noradrenaline cell bodies and into the nucleus raphe dorsalis or into the nucleus raphe medianus, two nuclei containing cell bodies of serotonergic neurones. Intraventricular administration of paraquat at a dose of 10 and 50 μ g caused intense behavioural stimulation and an increase in locomotor activity, circling and the wet-dog syndrome. This was accompanied by desynchronization of the electrocorticogram and the appearance of bilateral high voltage epileptiform spikes, and finally clonic convulsions occurred. The infusion of paraquat into the substantia nigra pars compacta (1 μ g) produced contralateral head and neck deviation, rigidity and kyphosis as well as behavioural and motor stimulation. The electrocorticogram activity was desynchronized and characterized by high voltage spike discharges. A similar behavioural, postural and electrocorticogram pattern was seen after infusion of paraquat into the caudate nucleus (10, 25 and 50 μ g). In addition, paraquat, infused into the locus coeruleus or into the raphe nuclei (5 and 10 μ g), produced circling, escape responses, jumping and clonic convulsions accompanied by electrocorticogram desynchronization and epileptic phenomena. The authors concluded that paraquat was able to produce central neurotoxicological effects that did not seem to be specific, at least for the doses used, for the dopamine nigro-striatal system (Gori et al., 1988).

In a study of the pathological effects of paraquat when administered directly into different parts of the rat brain, the microinfusion of paraquat (3.2, 16, 32 or 160 nmol) into the pars compacta of the substantia nigra produced neuropathological changes culminating in neuronal necrosis. A particular feature of paraquat neurotoxicity after its microinfusion into the substantia nigra (3.2 mmol/l at 1 μ l/min for 1 min) or into the ventral tegmental area (1.6 mmol/l at 1 μ l/min for 1 min), but not into other areas of the brain, was selective vulnerability of hippocampal CA3 neurones. This initially comprised a decrease in dendritic spines, which was followed by neuronal degeneration and cell loss. No damage occurred

after microinfusion of paraquat into other areas of the brain near or distant from the infusion sites. In addition, similar neuropathological alterations occurred in other non-dopaminergic areas. The authors considered that the study showed that paraquat possesses marked neurotoxicity that is not selective for dopaminergic neurones (Calò et al., 1990).

In a study of the effects of injected MPTP and analogues of MPTP *inter alia*, paraquat and reduced paraquat, C57 black mice were given paraquat in three subcutaneous injections of 14.5 mg/kg bw at an interval of 3 days, each injection being at a maximum tolerated dose. Reduced paraquat was administered in six daily doses increasing from 7.3 to 116.3 mg/kg bw, with a total dose of 342 mg/kg bw; this dose was well tolerated. One month after the last injection with paraquat or reduced paraquat, striatal dopamine was not depleted, while it was severely reduced with MPTP (Perry et al., 1986).

In a study investigating the possible role of paraquat in Parkinson disease, paraquat or MPTP were administered intraperitoneally to groups of six adult C57 Bl/6 mice. The dosing regimen for paraquat was 5 or 10 mg/kg bw given as three injections at weekly intervals, while that for MPTP was 10 or 30 mg/kg administered at 7 days and 16 h later and at 15 days and 16 h later (*i.e.* four doses). Saline was administered to a control group of six mice. Ambulatory behaviour was monitored. Substantia nigra dopamine neurone number and striatal dopamine terminal density were quantified after death. The data indicated that paraquat elicited a dose-dependent decrease in substantia nigra dopaminergic neurones (assessed by a fluoro-gold prelabelling method), a decline in striatal dopamine nerve terminal density (assessed by measurement of tyrosine hydroxylase immunoreactivity), and a neurobehavioural syndrome characterized by reduced ambulatory activity. Similar findings were seen with MPTP. The authors suggested that systemically absorbed paraquat crossed the blood-brain barrier to cause destruction of dopamine neurones in the substantia nigra and reduction of dopaminergic innervation of the striatum. The use of a parenteral route of administration, however, means that these data are of questionable relevance for risk assessment of paraquat residues (Brooks et al., 1999).

In a study of neurotoxic effects after neonatal exposure to paraquat and MPTP, groups of mice (aged 10 or 11 days) were given vehicle (water), paraquat, or MPTP by mouth; MPTP was administered at a dose of 0.3 or 20 mg/kg bw, and paraquat at a dose of 0.07 or 0.36 mg/kg bw. Neonatal spontaneous motor activity was tested on day 18 in mice given paraquat at 0.36 mg/kg bw. Adult spontaneous motor activity was tested at ages 60 and 120 days. On day 125, the mice were decapitated and the contents of dopamine and serotonin and metabolites in striatum were analysed. Acute toxicity was not observed in any of the groups. No respiratory distress or motor performance dysfunction was seen on day 18 in mice given paraquat at 0.36 mg/kg bw. The results of behavioural tests carried out at age 60 days showed a marked hypoactive condition in the mice given paraquat (at both doses) and MPTP (at both doses). At age 120 days, the hypoactivity persisted and appeared even more pronounced. Reduced striatal content of dopamine and metabolites was seen in the striatum with both compounds, but concentrations of serotonin were unaffected. The effect was greater at the higher doses (Fredriksson et al., 1993).

In a study in two strains of mice, one (C57 black) being the same as that used by Fredriksson et al. (1993), paraquat was administered as single daily doses at 0.36 or 3.6 mg/kg bw to pups aged 10 or 11 days, and appropriate controls were used (Ray, personal communication, 2003). Testing for spontaneous behaviour was carried out at 4 months, and approximately 1 week later the mice were killed and analysed for neurotrans-

mitters in the brain, as well as muscarinic receptor density. In the C57 black mice at 4 months, there was hyperactivity at 0.36 mg/kg bw compared with the controls, while at 3.6 mg/kg bw and in the other strain of mice used (NMRI) at both doses there were no significant differences from the controls. There were no significant intergroup differences in muscarinic receptor density nor in striatum or hippocampus dopamine, metabolites of dopamine or 5-hydroxyindoleacetic acid. The authors concluded that, using similar conditions, they could not replicate the results of the Fredriksson et al. (1993) study.

In the study by Widdowson et al. (1996b) on the entry of paraquat into the brains of male Wistar-derived Alpk:Apfsd rats, discussed above, groups of four rats were dosed daily for 14 days with water (controls) or 5 mg of paraquat ion/kg bw, orally. The rats were killed 24 h after the last of the 14 doses or after the single dose. On days 4 and 12, open field testing was carried out. On day 15, activity was measured over 50 min using an animal activity monitor, while animal grip strength and coordination was tested on days 4, 8 and 15 of the study. The brains were processed for histopathological examination after fixation by intracardiac perfusion. Brain catecholamines were measured by high-performance liquid chromatography using electrochemical detection, while dopamine D₁ and D₂ receptors were labelled using ³H-labelled SCH23390 and spiperone respectively. The density of muscarinic acetylcholine receptors was estimated with ³H-*N*-methyl scopolamine, and of *N*-methyl-D-aspartate (NMDA) receptors by ³H MK-801 binding. The density of benzodiazepine sites on GABA_A receptors was measured by ³H-labelled Ro15-1788 binding. Body-weight gain was decreased in the test animals in comparison with the controls. No differences between the groups were seen in the results of behavioural tests. There was no sign of neuronal cell damage in the test group, in particular in the substantia nigra. The concentration of dopamine was significantly higher in the striatum of rats treated with paraquat than in controls, but this was not the case in the hypothalamus. Differences in D₁, D₂, muscarinic, *N*-methyl-D-aspartate and benzodiazepine sensitive GABA_A receptors was not seen. The authors concluded that paraquat did not behave in the same way as MPTP in the tests used (Widdowson et al., 1996b).

(e) *Possible neurotoxic interactions*

Thiruchelvam et al. (2000a, 2000b) carried out studies to assess the potential involvement of combined exposure to the herbicide paraquat and to maneb, a manganese-containing ethylenedisithiocarbamate fungicide, in the etiology of idiopathic Parkinson disease.

Male C57 Bl/6 mice were given paraquat dichloride at a dose of 5 or 10 mg/kg bw and/or maneb at a dose of 15 or 30 mg/kg bw, once weekly for a total of 4 weeks, by intraperitoneal injection. End-points assessed were: effects on locomotor activity, density of tyrosine hydroxylase positive neurones, concentrations of dopamine and metabolites, and dopamine turnover. The authors noted that decreases in motor activity immediately after injections were observed more consistently with combined exposures to maneb and paraquat. Concentrations of dopamine and metabolites and dopamine turnover were slightly increased immediately after injection of combined maneb and paraquat, compared with injection of maneb alone. In addition, significant reductions in tyrosine hydroxylase immunoreactivity, measured 3 days after the last injection, were detected in the dorsal striatum of animals given combined treatments, but not those treated with single compounds. The authors concluded that these results demonstrated potentiating effects of combined exposures to paraquat and maneb on nigrostriatal dopamine systems (Thiruchelvam et al., 2000a).

In similar experiments, male C57 Bl/6 mice were given single compounds (paraquat at a dose of 10 mg/kg bw or maneb at a dose of 30 mg/kg) or a combination (paraquat at 10 mg/kg bw paraquat plus maneb at 30 mg/kg bw), twice weekly by intraperitoneal injection for 6 weeks. It was reported that maneb, but not paraquat, reduced motor activity immediately after treatment, and that this effect was potentiated by combined treatment with paraquat and maneb. As treatments progressed, only the groups receiving combined paraquat and maneb showed a failure of motor activity recovery within 24 h. Paraquat and maneb in combination, but not alone, reduced tyrosine hydroxylase and dopamine transporter immunoreactivity in the dorsal striatum, but not in the nucleus accumbens. Reactive gliosis occurred only in response to combined paraquat and maneb in dorsal-medial but not ventral striatum. Tyrosine hydroxylase immunoreactivity and cell counts were significantly reduced only by the mixture of paraquat and maneb, and not by the pesticides alone, in the substantia nigra, while no treatment produced significant effects on tyrosine hydroxylase immunoreactivity and cell counts in the ventral tegmental area. The authors suggested that the combination of paraquat and maneb showed synergistic effects, preferentially expressed in the nigrostriatal dopamine system, and suggested that such mixtures could play a role in the etiology of Parkinson disease. The study was not designed appropriately to investigate potentiation and the results could have reflected dose-additivity (Thiruchelvam et al., 2000b).

(f) *Experimental therapies*

Among treatments for poisoning with paraquat that have been studied in experimental animals is the injection of the enzyme superoxide dismutase. Steroids have also been studied (Kitazawa et al., 1988; Chen et al., 2003), without apparent benefit. This appeared to be beneficial in rats that had been given paraquat administered by gavage (Autor, 1974). The results of studies in rats suggested that paraquat might potentiate the toxic effects of oxygen (Fisher et al., 1973; Keeling et al., 1981). Prolonged (6 h) haemoperfusion was reportedly successful in saving three out of four pigs (Landrace × Yorkshire gilts) to whom paraquat at a dose of 70 mg/kg bw had been administered by stomach tube. This dose was fatal in untreated pigs and 2 h of haemoperfusion was ineffective. The purity of the paraquat used was not stated, nor is it clear whether the dose was expressed as paraquat ion or dichloride (Yang et al., 1997).

(g) *Poisoning of animals*

Paraquat poisoning in animals is rare (Blood et al., 1983). Nevertheless, from time to time paraquat is reported as the causative agent in animal poisoning, Longstaffe et al. (1981), for example, reported malicious and accidental poisoning of cats and dogs, and Aleksic-Kovacevic et al. (2003) reported the accidental poisoning by paraquat of five German shepherd dogs.

3. Observations in humans

3.1 *Poisoning incidents*

Intentional ingestion of paraquat is a major cause of death from poisoning. Casey & Vale (1994) tabulated deaths from pesticide poisoning from 1945–1989 in England and Wales and found that paraquat was responsible for 570 deaths, or 56.3% of all deaths caused by pesticides. From 1982, however, there has been a progressive decline in the annual number of poisonings after the inclusion of emetic, stench and dye into gramoxone formulations.

There are numerous case reports and case series of poisonings with paraquat (e.g. Bullivant, 1966; Campbell, 1968; Malone et al., 1971; Douze et al., 1974; Carson & Carson, 1976; Bismuth et al., 1982; Bramley & Hart, 1983; Naito & Yamashita, 1987; Wesseling et al., 1993; Hall, 1995; Tsatsakis et al., 1996; van Wendel de Joode et al., 1996; Wesseling et al., 1997; Papanikolaou et al., 2001). The effects can be divided into local and systemic effects. Local effects may comprise damage to the skin, nails, and nose (Samman & Johnston, 1969; Hearn & Keir, 1971; Vale et al., 1987; Bismuth et al., 1995), and sore throat, dysphagia and epigastric pain may also occur. Local effects to the eye may heal only slowly and with scarring (Peyresblanques, 1969; Devečková et al., 1980). After ingestion of formulation concentrate, ulceration of the upper gastrointestinal tract is often observed. Although these effects are unpleasant, the findings from those poisonings with a fatal outcome are generally referable to the respiratory system, death being preceded by dyspnoea and cyanosis. Crepitations may be heard. Radiology initially reveals diffuse fine mottling of the lungs. Renal dysfunction may partly be a direct effect of paraquat and partly be caused by hypovolemia; often mild, renal dysfunction impairs the only route of elimination available (Marrs & Proudfoot, 2003). Lung function tests are commonly abnormal (Bismuth et al., 1982). At autopsy, there may be a pleural effusion, and damage to the upper respiratory tract. Grossly, the lungs appear solid, with haemorrhages, including subpleural ones. Histologically, there is oedema and the alveoli are airless with fibroblastic proliferation in the alveolar walls. Infiltration with mononuclear cells, polymorphs, macrophages and eosinophils has been reported. The longer the survival time, the greater the proliferation of epithelium and fibroblasts in the alveoli (Carson & Carson, 1976). Tubular damage in the kidney has been reported as well as mid-zonal and centrilobular degeneration in the liver. Proudfoot et al. (1979) reported that the plasma concentration of paraquat was a good predictor of the outcome in that persons whose plasma concentrations were below 2.0, 0.6, 0.3, 0.16 and 0.1 mg/l at 4, 6, 10, 16 and 24 h respectively after ingestion survived. Scherrmann et al. (1987) reported that plasma concentrations of paraquat in persons admitted more than 24 h after poisoning were predictive of the outcome of the poisoning in most patients. Furthermore, they concluded, on the basis of study of 53 patients, that persons with urinary concentrations of paraquat of <1 mg/l within 24 h of exposure would survive, while a fatal outcome could be anticipated in most persons in whom the urinary concentration of paraquat was >1 mg/l.

In a fatal case of paraquat poisoning in a pregnant woman, who developed the typical symptoms and signs of paraquat poisoning and at postmortem had the typical lung pathology of paraquat poisoning, the fetal lungs were normal (Fennelly et al., 1968). Talbot & Fu (1988), however, who reported the details of nine pregnant women who deliberately ingested paraquat, stated that paraquat in one case was concentrated 4–6 times in the fetus. In another of the cases, the amniotic fluid contained paraquat at twice the concentration of that in the maternal blood. All the fetuses died, whether or not caesarian section was carried out. A case of paraquat poisoning in early pregnancy was reported from French Guiana. A woman who was 10 weeks pregnant took Grammoxone®, in a suicide attempt. She developed oliguria and underwent dialysis. The blood concentration of paraquat was 0.22 mg/l. No pulmonary symptoms or signs were noted and renal function progressively returned to normal. The woman gave birth normally at 39 weeks and both mother and baby remained well during 4 years of follow-up (Raynal et al., 2003).

Although most patients who have radiological lung changes go on to develop progressive and ultimately fatal lung damage, there are a few case reports in which patients have developed persistent radiological changes but have survived (e.g. Hudson et al., 1991).

There is also evidence that, in such patients, some recovery may occur over time (Ming et al., 1980; Lin et al., 1995; Papiris et al., 1995).

It has been reported that alcohol may increase the severity of paraquat poisoning (Ernouf et al., 1998), but the reverse has also been suggested (Ragoucy-Sengier et al., 1991).

The vast majority of paraquat intoxications are by ingestion. Athanaselis et al. (1983), however, reported the poisoning via the skin of a 64-year-old spray operator. Fluid had leaked down his back for several hours, causing irritation of the skin. Two days later, the spray operator visited a doctor, who advised hospitalization. The patient rejected this advice, but was admitted into hospital 3 days later. He died of toxic shock and renal and respiratory insufficiency 12 h after admission. At autopsy, the findings were typical of paraquat poisoning with fibrosing interstitial pneumonitis and intra-alveolar haemorrhage in the lungs, renal tubular cell degeneration, cholestasis and necrosis of the skin of the back. A further case of a fatality from transdermal exposure to paraquat was reported from Papua New Guinea, the patient evidently thinking that Gramoxone (20% paraquat w/v) would kill lice, for which purpose he applied the material to his scalp and beard. This produced painful sores and his condition steadily deteriorated until death 6 days after applying the paraquat to his skin. At autopsy, there were skin lesions as well as solid and haemorrhagic lungs (Binns, 1976). In a further report, Garnier et al. (1994) reported two cases of percutaneous exposure. In the first case, a man aged 36 years applied 20% paraquat concentrate to his whole body to cure scabies. He developed extensive erythema followed by blistering and 2 days later he was admitted to hospital. He developed transient renal failure. Dyspnoea appeared 1 week after admission and he deteriorated, dying 26 days after exposure. The other case reported by Garnier et al. (1994) was much milder, with mainly skin effects, and the outcome was not fatal. Additional cases of fatal percutaneous paraquat intoxication were reported by Newhouse et al. (1978), Levin et al. (1979), Wohlfahrt (1982), Okonek et al. (1983) and Papiris et al. (1995). In general, systemic toxicity after percutaneous exposure of humans seems to be unusual (Hoffer & Taitelman, 1989).

There is evidence that as well as the route of exposure, the formulation may be important in determining the severity of effects. A case series of 14 instances of poisoning with granular paraquat and diquat at low concentrations was reported by Fitzgerald & Barniville in 1978. No deaths occurred, the illness was mild and necrotic lesions of the mouth and pharynx were not seen.

Initial management of cases of poisoning with paraquat comprises replacement of fluid loss, determination of the prognosis by measurement of the plasma concentration of paraquat, treatment of local damage to the oropharynx, and supportive care (Vale et al., 1987).

Numerous treatments have been tried in the management of cases of poisoning with paraquat, many concentrating on the prevention of absorption (Meredith & Vale, 1987). Gastric lavage, fuller's earth and activated charcoal have all been tested: other therapies that have been investigated include removal of paraquat from the blood by forced diuresis, peritoneal dialysis, haemodialysis or haemoperfusion using sorbent materials, including charcoal haemoperfusion (Tabei et al., 1982). Corticosteroids have also been tried (Bismuth et al., 1982; Chen et al., 2002), as have acetylcysteine and deferoxamine (Lheureux et al., 1995), and *S*-carboxymethylcysteine (Lugo-Vallin et al., 2003) and radiotherapy (Talbot & Barnes, 1988). Addo et al. (1984) reported that treatment with cyclophosphamide, dexam-

ethasone, forced diuresis with frusemide, triamterine and hydrochlorothiazide enabled the survival of 15 of 20 patients. This therapy was combined with routine measures, such as fuller's earth, activated charcoal and magnesium sulfate to eliminate paraquat from the gut. Time has, however, shown that none of the measures discussed above are consistently successful, therefore treatment is perforce symptomatic (Vale et al., 1987). The use of oxygen may increase the severity of pulmonary fibrosis (Bismuth et al., 1982) and should be delayed as long as possible. The therapy of paraquat poisoning has been reviewed (Flanagan & Jones, 2001).

3.2 *Epidemiological studies*

In an analysis of all cases of early onset Parkinson disease in persons born and raised in Saskatchewan, Canada, it was found that 20 out of 22 cases were exclusively exposed to a rural environment during the first 15 years of life. This distribution was significantly different from that of the general population ($p = 0.0141$). Further study included sampling and metal analysis of sources of drinking-water in childhood in 18 cases and in 36 age- and sex-matched controls. Drinking-water to which the individuals in the cases and controls had been exposed was collected and analysed for 23 metals. There was no difference in the metal composition of the water between the two groups. A review of pesticide usage from Saskatchewan agricultural records was undertaken to determine if there was an increased incidence of early onset Parkinson disease after use of any particular chemical. No increase was found in the incidence of the disease with the introduction of any pesticide, including paraquat, for agricultural use (Rajput et al., 1987).

In a case-control study, the personal histories of 57 cases and 122 age-matched controls were compared to identify possible determinants of Parkinson disease. Odds ratios (OR) adjusted for sex, age, and smoking were computed using stepwise logistic regression. A statistically significant increased risk for working in orchards was found (OR, 3.69; 95% CI, 1.34–10.27; $p = 0.012$). The relative risk of Parkinson disease decreased with smoking, an inverse relationship that was supported by the results of many studies (Hertzman et al., 1990).

A questionnaire-based case-control study to investigate possible risk factors for Parkinsonism involved 150 patients with Parkinson disease and 150 controls matched by age and sex. Use of well water and rural living were associated with Parkinsonism, but farming and pesticide/herbicide use was not (Koller et al., 1990).

In a case-control study of 130 cases of Parkinson disease and 260 age- and sex-matched controls from Calgary, Alberta, Canada, no significant association of Parkinson disease with rural or farm living or drinking well water in early childhood was found (Semchuk et al., 1991).

A retrospective case-control study, with 127 cases and 245 controls was carried out to identify possible risk factors for idiopathic Parkinsonism. Of the controls, 121 had cardiac disease and 124 were randomly selected from electoral lists. An occupational history was collected, and known contact with all pesticides associated with the tree-fruit sector of the agricultural industry was recorded. There was a significant association between Parkinsonism and having had an occupation in which exposure through handling or directly contacting pesticides was probable, but no specific chemicals were associated with the condition. The authors concluded that although occupations involving the use of agricultural chemi-

cals might predispose to the development of Parkinsonism, it was likely that the pathogenesis is multifactorial rather than related to a specific agent (Hertzman et al., 1994).

In a cross-sectional study undertaken in the Republic of Nicaragua to evaluate any relationship between respiratory health and paraquat exposure, the study population was selected from among workers at 15 banana plantations that used paraquat as a herbicide. All workers who reported never having applied paraquat and all who reported more than 2 years of cumulative exposure as knapsack sprayers of paraquat were invited for medical examination. There were 134 workers in the group that was exposed to paraquat and there were 152 workers that were not exposed. All took part in a questionnaire interview asking about exposure and respiratory symptoms, and underwent spirometric testing of forced expiratory volume in 1 s (FEV_{1.0}) and forced vital capacity (FVC). Of the persons in the exposed group, 53% reported having experienced a skin rash or burn resulting from exposure to paraquat, 25% reported epistaxis, 58% reported nail damage, and 42% reported paraquat splashes to the eyes. There was a consistent relationship between a history of skin rashes or burns and the prevalence of dyspnoea. This relationship was more marked for more severe dyspnoea. There was a three-fold increase in episodic wheezing accompanied by shortness of breath among the more intensely exposed workers. There was no relationship between exposure and FEV_{1.0} or FVC. The authors considered that the high prevalence of respiratory symptoms associated with exposure, in the absence of spirometric abnormalities associated with exposure, could be a result of unmeasured gas exchange abnormalities among workers with long-term exposure to paraquat. They could also have been caused by recall bias (Castro-Gutiérrez et al., 1997).

3.3 *Studies in human volunteers*

A study of the percutaneous absorption of paraquat in vivo was undertaken in six human volunteers by Wester et al. (1984). ¹⁴C-Labelled paraquat dichloride at a dose of 9 µg/cm² was applied to a 70 cm² area of the skin of the back of the leg, the back of the hand or the ventral surface of the forearm. The specific activity of the paraquat was 2.0 mCi per mmol per l and the concentrations of the solution were given as paraquat dichloride, not as paraquat ion. Urine samples were collected at 4, 8, 12, and 24 h, and then every 24 h for 5 days. The extent of percutaneous absorption was measured by comparing the excretion of ¹⁴C after parenteral and topical administration; rather than administer the paraquat to humans, it was administered to rhesus monkeys. The percentage of the applied dose that was absorbed was 0.29 ± 0.2 (mean ± SD) for the leg, 0.23 ± 0.1 for the hand and 0.29 ± 0.1 the forearm. The absorption rate for the 24 h of exposure was 0.03 µg/cm². It was concluded that paraquat was poorly absorbed through human skin and that there was little difference between skin at different sites in ability to absorb paraquat.

Comments

The pharmacokinetics and metabolism of paraquat have been the subject of many studies. Paraquat is not well-absorbed when administered orally. After oral administration of radiolabelled paraquat to rats, more than half the administered dose (60–70%) appeared in the faeces and a small proportion (10–20%) in the urine. In studies involving single or repeated doses, excretion of the radiolabel was rapid; about 90% was excreted within 72 h. Residual radioactivity was primarily found in the lungs, liver and kidneys. Some studies have found small amounts in the brain, but only in structures outside the blood–brain barrier or in structures without a blood–brain barrier (the pineal gland and linings of the

cerebral ventricles, the anterior portion of the olfactory bulb, hypothalamus and area postrema). Paraquat is taken up into the lungs by an active process, whose normal substrate is endogenous diamines, e.g. putrescine and polyamines such as spermine and spermidine. In rats, dogs and monkeys, there are indications that paraquat is actively secreted in the kidneys.

Paraquat is largely eliminated unchanged; in rats, approximately 90–95% of radiolabelled paraquat in urine was excreted as the parent compound. Some studies have failed to show the presence of any metabolites after oral administration of paraquat, while others have shown a small degree of metabolism, which probably occurs in the gut as a result of microbial metabolism. Paraquat was not found in the bile.

The acute LD₅₀ after oral administration was 290–360 mg/kg bw in mice and 112–350 mg/kg bw in rats, while the guinea-pig was more sensitive (LD₅₀ of 22–30 mg/kg bw). The LD₅₀ in cynomolgus monkeys was 50–70 mg/kg bw. Paraquat was considered to be a mild skin irritant and a moderate ocular irritant and was not a skin sensitizer in the Magnusson and Kligman test.

The predominant feature of exposure to repeated doses of paraquat was lung toxicity. Renal toxicity (proximal tubular damage) and toxicity to the liver (jaundice and elevations of enzyme activity) were also found. In some studies, lens opacities were seen. At higher doses, decreased body-weight gain, clinical signs (dyspnoea, increased respiratory sounds, swellings and sores in the genital area), haematological changes and effects on organ weight were reported, as well as increased mortality.

Lung abnormalities observed in mice, rats and dogs consisted of increased lung weight and gross pathological changes. Associated histopathological changes included cell necrosis, alveolar cell proliferation and hypertrophy, oedema, infiltration of macrophages and mononuclear cells and exudate. Dogs were most sensitive to paraquat-induced lung toxicity, followed by rats and mice; a NOAEL of 0.45 mg of paraquat ion/kg bw per day was found in a 1-year study in dogs, on the basis of signs of respiratory dysfunction and histopathological changes at higher doses. This finding was supported by the NOAEL of 0.55 mg of paraquat ion/kg bw per day from a 13-week study in dogs.

Ophthalmoscopy in-life and histopathological examination of eyes at necropsy revealed corneal opacity and cataracts in animals receiving doses of 3.75 mg and 7.5 mg of paraquat ion/kg bw per day in a lifetime study in Fischer rats. Other ocular effects included lenticular degeneration, lens capsular fibrosis and/or lens ruptures, peripheral retinal degeneration, and proteinaceous vitreous humour. At time-points after 2 years (i.e. after the study would have ended according to current guidelines), rats receiving the lowest dose exhibited age-related peripheral morgagnian corpuscles and slight peripheral and moderate mid-zonal lenticular degeneration. Histopathological evidence of cataracts was also found at the highest dose (7.67 mg of paraquat ion/kg bw per day) in a 2-year study in Fischer rats, but not at lower doses. In another 2-year study in Wistar rats, no intergroup differences in the prevalence of cataracts were seen. These differences between effects on the lens in the three long-term studies in rats may be indicative of a difference between Wistar and Fischer rats.

Paraquat elicited renal toxicity, which comprised changes in the proximal tubules of the kidneys (hydropic degeneration, eosinophilia and dilatation) in mice fed with 15.0 mg of paraquat ion/kg bw per day in a lifetime study. Some very mild changes were also

observed in males at 5.62 mg of paraquat ion/kg bw per day, however, there was a clear NOAEL at 1.88 mg of paraquat ion/kg bw per day. There were some histopathological effects on renal distal tubular cells at 1.75 mg and 3.52 mg of paraquat ion/kg bw per day in a 13-week study in dogs, the NOAEL being 0.55 mg of paraquat ion/kg bw per day.

The frequency of pulmonary adenoma was increased in females in a 2-year study in rats receiving a dose of 8.47 mg of paraquat ion/kg bw per day; however, there was a clear NOAEL at 3.13 mg of paraquat ion/kg bw per day. In males, adenocarcinoma was found in three animals (out of 80) receiving a dose of 10.6 mg of paraquat ion/kg bw per day, one animal (out of 80) receiving 3.52 mg of paraquat ion/kg bw per day and two animals (out of 80) receiving 1.34 mg of paraquat ion/kg bw per day. The NOAEL for males in this study was 0.77 mg of paraquat ion/kg bw per day on the basis of histopathology of the lungs. In a second 2-year study in rats, no intergroup differences in tumour incidence were seen at any site. After review of the histopathological findings in the lifetime study in rats, it was concluded that the incidence of lung neoplasms in the test groups was comparable to that in the control groups. Thus tumours were seen in only one out of three long-term studies in rats. The Meeting concluded that the weight of evidence suggested that paraquat was not carcinogenic in the rat. Paraquat was not considered to be tumorigenic in two studies in mice.

Paraquat has been tested extensively in a broad range of assays for genotoxicity *in vitro* and *in vivo*, with mixed results. Studies more commonly gave positive results when DNA damage or clastogenicity were the end-points. Paraquat is known to produce active oxygen species and the available evidence indicates that it is probably this property that is responsible for its genotoxicity. Consequently, there is a threshold below which genotoxic activity will not be evident, provided that normally functioning antioxidant defence mechanisms have not been overwhelmed. The Meeting concluded that paraquat is unlikely to pose a genotoxic risk to humans.

Because of the nature of the genotoxicity observed and the lack of carcinogenicity in rats and mice, the Meeting concluded that paraquat was unlikely to pose a carcinogenic risk to humans.

Three studies of reproductive toxicity in rats were reported. The overall NOAEL for parental toxicity was 1.67 mg of paraquat ion/kg bw per day, and the NOAEL for pup toxicity was 5.0 mg of paraquat ion/kg bw per day. Impaired fertility was not seen in these studies. Two studies of developmental toxicity in rats and two in mice were available for evaluation. The lowest NOAELs observed for both maternal and developmental toxicity in rats were 1 mg of paraquat ion/kg bw per day on the basis of clinical signs, and reduced body-weight gain in the dams and reduced mean fetal weights and retarded ossification in the fetuses. Higher NOAELs for maternal and developmental toxicity were seen in mice. Teratogenicity was not seen at any dose in any study in either rats or mice.

Paraquat is structurally similar to the known dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). As a result, paraquat has been considered as a possible etiologic factor in Parkinson disease. However, paraquat is a quaternary nitrogen compound and therefore crosses biological membranes poorly, unlike MPTP, the precursor of the neurotoxicant methylphenylpyridinium ion. Data made available to the Meeting suggested that paraquat is not taken up by the dopamine transporter. Studies on the effects of paraquat on the central nervous system have used a variety of routes, including subcuta-

neous or intraperitoneal injection and direct injection into the central nervous system, and end-points observed have been behavioural, morphological and neurochemical. Behavioural effects and loss of neurones in the substantia nigra were observed and, neurochemically, depletion of dopamine was reported in many, but not all of these studies. The design of these studies, however, renders the relevance of these data questionable for the risk assessment of dietary exposure to paraquat residues.

Persistent hypoactivity was observed in mice given paraquat by mouth on postnatal days 10 and 11. Reduced striatal content of dopamine and its metabolites was seen, but concentrations of serotonin were not affected. In a similar study of which the Meeting was aware, these findings had not been reproduced.

The Meeting concluded that the available mechanistic and other animal studies did not support the hypothesis that paraquat residues in food are a risk factor for Parkinson disease in humans.

Two studies carried out to assess the potential involvement of combined exposure to paraquat and maneb, a manganese-containing ethylenebisdithiocarbamate fungicide, in the etiology of idiopathic Parkinson disease were evaluated by the Meeting. Paraquat or maneb, or a combination of the two, was given intraperitoneally to mice. The study was not designed appropriately to investigate potentiation and the results could have reflected dose-additivity.

Intentional and accidental poisonings with paraquat have been a major cause of death in many countries. Most incidents are caused by ingestion of the concentrate intended for agricultural use. Local effects include damage to the skin, nails, mouth, eyes and nose. Sore throat, dysphagia and epigastric pain may occur. Systemic effects, which produce the fatal outcome seen in those who have ingested a sufficient quantity of paraquat, mainly involve the respiratory system. The changes in the lungs that underly the symptoms and clinical signs comprise a proliferative alveolitis similar to that seen in most experimental animals treated with paraquat. In most, but not all, patients who develop the characteristic lung changes, the condition progresses inevitably towards a fatal outcome, death being due to respiratory failure. Numerous therapies have been tested, but none has been consistently successful.

A number of epidemiological (case-control) studies have been carried out in humans with Parkinson disease. In some of these, associations with exposure to chemicals including pesticides (in some cases specifically paraquat) were sought. Some but not all studies have shown a relationship between working in situations that might involve contact with or use of pesticides and Parkinson disease, but associations with exposure to specific pesticides have not been shown consistently.

The Meeting established an ADI of 0–0.005 mg of paraquat ion/kg bw based on a NOAEL of 0.45 mg of paraquat ion/kg bw per day in the 1-year study in dogs and using a safety factor of 100. Although a 1-year study in dogs is not considered to be a long-term study, the nature and time-course of the pathogenesis of the lung lesions were such that the application of an additional safety factor was not considered to be necessary.

The Meeting established an acute RfD of 0.006 mg of paraquat ion/kg bw based on the NOAEL of 0.55 mg of paraquat ion/kg bw per day in the 13-week study in dogs, with

a safety factor of 100. Histopathological changes in the lungs were present at higher doses in both studies in dogs.

Toxicological evaluation

Levels relevant to risk assessment

Species	Study	Effect	NOAEL ^a	LOAEL ^a
Mouse	13-week study	Toxicity	100 mg/kg, equal to 8.33 mg of ion/kg bw per day	300 mg/kg, equal to 25.9 mg of ion/kg bw per day
	97–99-week study	Toxicity	12.5 mg/kg, equivalent to 1.88 mg of ion/kg bw per day	37.5 mg/kg, equivalent to 5.62 mg of ion/kg bw per day
		Carcinogenicity	100 mg/kg equivalent to 15.0 mg of ion/kg bw per day ^b	—
	Study of developmental toxicity	Maternal toxicity	10 mg/kg bw per day ^b	—
		Embryo- and fetotoxicity	10 mg/kg bw per day ^b	—
Rat	13-week study	Toxicity	100 mg/kg, equal to 4.74 mg/kg bw per day	300 mg/kg, equal to 14.2 mg/kg bw per day
	104-week study	Toxicity	30 mg/kg, equal to 0.77 mg/kg bw per day	100 mg/kg, equal to 2.55 mg/kg bw per day
		Carcinogenicity	300 mg/kg, equal to 7.67 mg of ion/kg bw per day ^b	—
	Multigeneration study of reproductive toxicity	Parental toxicity	25 mg/kg, equivalent to 1.67 mg/kg bw per day	75 mg/kg, equivalent to 5.0 mg/kg bw per day
		Pup toxicity	75 mg/kg, equivalent to 5.0 mg/kg bw per day	150 mg/kg, equivalent to 10.0 mg/kg bw per day
	Study of developmental toxicity	Maternal toxicity	1 mg/kg bw per day	5 mg/kg bw per day
		Embryo- and fetotoxicity	1 mg/kg bw per day	5 mg/kg bw per day
Dog	13-week study	Toxicity	20 mg/kg, equal to 0.55 mg/kg bw per day	60 mg/kg, equal to 1.75 mg/kg bw per day
	1-year	Toxicity	15 mg/kg, equal to 0.45 mg/kg bw per day	30 mg/kg, equal to 0.93 mg/kg bw per day

^a Dietary concentrations are expressed as dichloride or paraquat ion as in the study report; intakes and doses are expressed as paraquat ion

^b Highest dose tested

Estimate of acceptable daily intake for humans

0–0.005 mg of paraquat ion/kg bw

Estimate of acute reference dose

0.006 mg of paraquat ion/kg bw

Studies that would provide information useful for continued evaluation of the compound

Further observations in humans

Summary of critical end-points for paraquat

Absorption, distribution, excretion and metabolism in mammals

Rate and extent of oral absorption	Poor
Dermal absorption	Poor; 0.25–0.29% absorbed (humans)
Distribution	Highest concentrations found in the lungs, liver and kidneys
Potential for accumulation	No potential for passive accumulation; active uptake into type II pneumocytes
Rate and extent of excretion	Rapid, about 64% in 24 h; 10% in urine, the remainder in the faeces; none is found in bile
Metabolism	Some metabolism (<5%) in gut (probably microbial); paraquat is largely excreted unchanged
Toxicologically significant compounds (animals, plants and environment)	Parent compound

Acute toxicity

Rat, LD ₅₀ , oral	100–300 mg paraquat ion/kg bw
Rat, LD ₅₀ , dermal	80–660 mg of paraquat ion/kg bw
Rat, LC ₅₀ , inhalation	0.0006–0.0014 mg of paraquat ion/l (4 h exposure)
Rabbit, skin irritation	Mild
Rabbit, eye irritation	Moderate
Skin sensitization	Not sensitizing (Magnusson and Kligman test)

Short term toxicity

Target organ/critical effect	Lung toxicity
Lowest relevant oral NOAEL	0.55 mg of paraquat ion/kg bw per day (13-week study in dogs); 0.45 mg of paraquat ion/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	1.15 mg of paraquat ion/kg bw per day (21-day study in rabbits)
Lowest relevant inhalation NOAEL	0.00001 mg/l (21-day study in rats)

Genotoxicity

Paraquat was clastogenic at high concentrations
Unlikely to pose a genotoxic risk to humans at dietary concentrations

Long term studies of toxicity and carcinogenicity

Target organ/critical effect	Lung toxicity
Lowest relevant NOAEL	0.77 mg of paraquat ion/kg bw per day (2-year study in rats)
Carcinogenicity	Not carcinogenic; unlikely to pose a carcinogenic risk to humans

Reproductive toxicity

Reproduction target/critical effect	Lung toxicity in pups
Lowest relevant reproductive NOAEL	5 mg of paraquat ion/kg bw per day (three-generation study in rats)
Developmental target/critical effect	Not teratogenic; reduced fetus weight and ossification at maternally toxic dose
Lowest relevant developmental NOAEL	1 mg of paraquat ion/kg bw per day (rats)

Neurotoxicity/delayed neurotoxicity

Not neurotoxic by oral route

Other toxicological studies

Mechanistic studies on lung, liver and kidney toxicity

Medical data

Causes acute poisoning

Summary	Value	Study	Safety factor
ADI	0–0.005 mg/kg bw	Dog, 1-year study	100
Acute RfD	0.006 mg/kg bw	Dog, 13-week study	100

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PHOSMET (addendum)

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Explanation

Phosmet (*O,O*-dimethyl *S*-phthalimidomethyl phosphorodithioate) is an insecticide and acaricide that acts by inhibiting acetylcholinesterase activity. Phosmet was evaluated previously by the JMPR in 1978, 1979, 1994 and 1998 (Annex 1, references 30, 32, 71, 83). An ADI of 0–0.01 mg/kg bw was set in 1994. In 1998, an acute reference dose (RfD) of 0.02 mg/kg bw was set, based on a NOAEL of 2 mg/kg bw per day in a study of developmental toxicity in rabbits. By request of a WHO Member State and the European Union, the acute RfD for phosmet was reviewed by the present Meeting in 2003, instead of in 2004 as originally scheduled. A recent study in human volunteers and additional information on the study of developmental toxicity in rabbits were reviewed by the current Meeting.

Evaluation for acute reference dose

The Meeting considered the new data and reviewed previously submitted information of relevance to the establishment of an acute RfD.

1. Developmental toxicity

Rats

In reviewing a study of developmental toxicity in rats (Hodge, 1991), the 1994 Joint Meeting (Annex 1, reference 73) concluded that: “. . . 5 mg/kg bw per day was the NOAEL for maternal toxicity. As no teratogenic or fetotoxic effects were seen, the NOAEL for developmental toxicity was 15 mg/kg bw per day.” In this study, groups of 24 mated female Wistar rats received phosmet (purity, 96.4%) at a dose of 0, 5, 10 or 15 mg/kg bw per day by gavage. Signs typical of cholinergic toxicity were seen in dams at 15 mg/kg bw per day, with reduced maternal body-weight gain evident at 10 and 15 mg/kg bw per day.

In a published paper (Staples, 1976), it was reported that the administration of phosmet at a dose of up to 30 mg/kg bw per day to groups of CD rats did not cause any

fetotoxicity. The NOAEL for maternal toxicity was reported to be 10 mg/kg bw per day. The 1994 JMPR was unable to set a NOAEL, as no concurrent control group was used

Rabbits

In a range-finding study of developmental toxicity, groups of 10 New Zealand white rabbits were given phosmet (purity, 96.4%) at a dose of 0, 5, 10 or 15 mg/kg bw per day by gavage. The fetuses were examined only for external malformations and cleft palate. Maternal toxicity was seen at 15 mg/kg bw per day, but no malformations were reported (Pinto, 1991).

Groups of 20 inseminated female New Zealand white rabbits were given phosmet (purity, 96.4%) at a dose of 0, 2, 5 and 15 mg/kg bw per day in corn oil by gavage on days 7–19 of gestation (insemination was performed on day 1). The animals were observed for clinical signs, and body weight and food consumption were recorded at regular intervals. On day 30 of gestation, all surviving animals were sacrificed and uteri were examined for live fetuses and intra-uterine deaths. All fetuses were weighed, sacrificed, and examined for external and visceral abnormalities, sexed, eviscerated and stained for skeletal examination. Assays for cholinesterase activity were not performed in this study. The study complied with good laboratory practice (GLP) and OECD test guideline 414 (1981).

A total of six rabbits died or were sacrificed: two at 15 mg/kg bw per day; three at 5 mg/kg bw per day and one at 2 mg/kg bw per day. One of the animals receiving the highest dose was sacrificed after showing signs of cholinergic toxicity. Maternal toxicity was evident at 15 mg/kg bw per day (Table 1), with reduced body-weight gain during the period of treatment (13.5 g versus 51.8 g in controls) and occasional clinical signs typical of acetylcholinesterase inhibition (e.g. salivation). Food consumption was reduced by about 10% at 15 mg/kg bw per day at the start of treatment. There was no evidence of maternal toxicity at 5 mg/kg bw per day. Complete resorption of a litter and a reduction in litter size at 2 mg/kg bw per day were not dose-related and were considered to be chance findings. Increases in total external and visceral defects, delayed ossification and minor skeletal defects were evident at 5 and 15 mg/kg bw per day (Table 1), but overall rates were reported to be within the ranges for contemporary historical controls. There was no clear evidence for a treatment-related increase in any individual variation or malformation. The only statistically significant increase in an individual variation was in extreme flexion of the forepaw at 5 mg/kg bw per day, but this was not reproduced at 15 mg/kg bw. A similar absence of dose-response relationship was seen in the sites of altered ossification at 5 mg/kg bw per day (Table 1); a dose-response relationship would be expected because approximately 90% of the administered dose of phosmet was absorbed at 25 mg/kg bw per day. There is no consensus on whether alterations in ossification of fetal bones are a specific effect that can be produced by a single dose, or whether they are secondary to maternal toxicity, or both of these. It was considered that the fetal effects seen at 5 mg/kg bw per day were minimal and not clearly related to a single dose of phosmet. The parental and fetal toxicity seen at 15 mg/kg bw per day were considered to be related to inhibition of cholinesterase activity (Moxon, 1991).

2. Observations in humans

Groups of human volunteers were given phosmet (purity, 96%) or placebo (lactose BP) in a study that was approved by an ethics committee, used prior informed consent, and complied with the declaration of Helsinki. The study was a double-blind, randomized,

Table 1. Findings in rabbits given phosmet by gavage during days 7–19 of gestation

Parameter	Dose (mg/kg bw)				Historic controls# range (mean ± SD) as %
	0	2	5	15	
Maternal body-weight gain (g) on days 7–19	51.8	69.9	57.4	13.5	NR
Maternal body-weight gain (g) on days 7–10	−42.4	−51.3	−53.8	−72.9	NR
No. of pregnant dams	18	16	20	16	NR
No. of dams with live fetuses on day 30	18	14	17	15	NR
Maternal body-weight gain (g) on days 10–13	54.4	39.6	25.6	−2*	NR
Viable fetuses (total/mean per dam)	141/7.8	92/6.6	132/7.8	118/7.9	NR
Mean fetal weight (g)	42.8	46.6	42.2	41.6	NR
Fetuses with major defects (some have both skeletal and visceral) (%)	3 (2.1)	3 (3.3)	6 (4.5)	5 (4.2)	NR
No. of fetuses with major external or visceral defects (%)	3 (2.1)	2 (2.2)	6 (4.5)	5 (4.2)	NR
No. of fetuses with minor external or visceral defects (%)	6 (4.3)	6 (6.5)	13 (9.8)	12 (10.2)	NR
No. of fetuses with major skeletal defects	2 (1.4)	1 (1.1)	1 (0.7)	1 (0.8)	NR
No. of fetuses with minor skeletal defects (mean %)	50 (35.4)	42 (45.6)	62* (46.9)	56* (47.4)	35.2–55.8 (42 ± 7.6)
No. of fetuses with skeletal variations (mean %)	136 (96.4)	91 (98.9)	121 (91.6)	111 (94.1)	NR
Partially ossified odontoid					
Fetus (%)	55 (39)	39 (42)	49 (37)	68** (57)	24–58 (39 ± 13)
Litter (%)	16 (88)	10 (63)	14 (70)	14 (87)	46–100 (80 ± 16)
Unossified fifth sternebra					
Fetus (%)	4 (3)	5 (5)	7 (5)	15** (13)	3–20 (9 ± 5)
Litter (%)	2 (10)	5 (31)	5 (25)	6 (37)	7–61 (28 ± 16)
Unossified sixth sternebrae					
Fetus (%)	8 (6)	5 (5)	8 (6)	11 (9)	0–8 (6 ± 2)##
Litter (%)	4 (22)	5 (31)	6 (30)	9* (56)	0–36 (29 ± 10)
Seventh transverse process partially ossified					
Fetus (%)	1	1	5 (4)	0	0–2.2 (0.4 ± 0.7)
Litter (%)			3 (15)		0–16.7 (3 ± 6)
Fully ossified second lumbar vertebrae					
Fetus (%)	0	0	6 (5)	0	0–5 (1.7 ± 1.9)
Litter (%)			3 (16)		0–21 (8 ± 7)
Bipartite sixth sternebrae					
Fetus (%)	1	2/2	5 (4)	1	0–5 (1.3 ± 1.4)
Litter (%)			3 (15)		0–31 (10 ± 9)
Extreme flexion of forepaw—fetus (%)	0	0	4 (4)	1	0
(fetal/litter incidence)—litter (%)			4* (15)		
Pes score (measure of foot ossification)	1.09	1.03	1.06	1.19*	1.03–2.0 (1.5 ± 0.5)

From Moxon (1991)
NR, not relevant
Nine studies conducted during 1990–1993, except ##, six studies conducted during 1990–1991
p* < 0.05; *p* < 0.01

placebo-controlled protocol and subjects were selected, healthy men and women aged 18–50 years. Six men per group received a single oral dose of phosmet of 1, 2, or 4 mg/kg bw, and three men per group received placebo. Six women received a single oral dose of phosmet of 2 mg/kg bw, and three women received placebo. The test substances were administered orally in capsules with 150 ml of water, approximately 5 min after breakfast. The paired groups receiving phosmet or placebo were dosed sequentially: 1 mg/kg bw in men, 2 mg/kg bw in men, 4 mg/kg bw in men and then 2 mg/kg bw in women, groups receiving placebo being treated concurrently with groups receiving phosmet. Subjects were dosed in the sitting position and remained sitting until 4 h after dosing.

Subjects remained in the clinic for 48 h after dosing and returned for follow-up visits at 96 h and 168 h after dosing. Investigations included vital signs, 12-lead electrocardiogram (ECG), continuous single channel ECG (30 min before dosing until 4 h after dosing), urine analysis, haematology, clinical chemistry, oral temperature, adverse events and plasma and

erythrocyte cholinesterase activities. Samples for cholinesterase determination (three replicates per sample time) were taken on days -10, -8, -4, -2, -1 and -30 min (before dosing), and at 1, 2, 4, 8, 12, 24, 48, 96 and 168 h after dosing. All samples taken before dosing and those taken at 24, 48 and 96 h after dosing were done so in the morning, at the same time, if possible. One subject was removed from the study after breaking the study conditions, and was replaced.

The overall pattern of findings was similar in groups receiving placebo and in the groups receiving phosmet. Notable changes in values for an individual subject could usually be linked to results obtained before dosing. Reports of adverse events typical of cholinesterase inhibition were similar in groups receiving placebo and in groups receiving phosmet (Table 2). It should be noted that as part of the informed consent, subjects were told what potential side-effects might be expected. Two findings that were possibly related to treatment with phosmet were a dose-related decrease in mean serum glucose concentration and an inhibition of erythrocyte cholinesterase activity after 1 h and 4 h in males at 4 mg/kgbw (Table 3). There was no evidence for cholinesterase inhibition caused by treat-

Table 2. Summary of adverse events in volunteers given phosmet

	Dose (mg/kg bw)							
	0 (Placebo)		1		2		4	
	Males	Females	Males	Females	Males	Females	Males	Females
No. of subjects	10*	3	6	—	6	6	6	—
No. of subjects with adverse events (%)	2 (20%)	2 (67%)	0 (0%)	—	1 (17%)	3 (50%)	1 (17%)	—
No. of compound-related adverse events** (%)	1 (10%)	1 (33%)	0	—	1 (17%)	0	1 (17%)	—

From Cameron (1999)

* Includes subject who was removed, and the replacement

** Test compound-related adverse events are defined as those assessed by the investigator (before the study blind was broken) as having a potential relationship to administration of the test compound of “possibly related”, “probably related” or “definitely related”

Table 3. Changes in erythrocyte cholinesterase activity (% relative to individual baseline value before dosing) in volunteers receiving phosmet

Time point (h after dosing)	Value given	Dose (mg/kg bw)				Linear trend
		0 (Placebo)	1	2	4	
<i>Males</i>						
1	Adjusted means* (range)	4.39 (-8.7; +13.2)	0.37 (12.2; +10.4)	3.04 (-2.8; +8.4)	-4.44 (-19.3; +21.1)	0.068
2	Adjusted means*	2.51	3.25	-2.47	4.52	0.77
4	Adjusted means* (range)	-0.65 (-13.3; +13.2)	1.53 (-5.6; +7.2)	-1.33 (-9.4; +4.7)	-6.87 (-14.0; +1.1)	0.093
8	Adjusted means*	10.51	2.33	-0.67	12.70	0.38
12	Adjusted means*	7.57	-4.57	2.24	9.10	0.23
24	Adjusted means*	7.50	-4.51	1.14	0.24	0.34
48	Adjusted means*	6.47	0.29	1.40	2.69	0.59
96	Adjusted means*	-2.93	-6.98	-4.41	-5.50	0.74
168	Adjusted means*	1.97	-5.11	5.00	0.44	0.78
1-168	Range	-17.3; +35.5	-17.2; +10.4	-14.9; +13.7	-19.4; +27.4	
<i>Females</i>						
1-168	Range	-12.4; +17.6	—	-16.2; +26.8	—	—

From Cameron (1999).

From Cameron (1999)

* Represented adjusted means from repeated measures (analysis of variance, ANOVA)

ment with phosmet in females. The changes in glucose concentrations in men treated with phosmet (relative to values before dosing) were within the range of values for men receiving placebo, and all values for men and women (4.3–6.0 mmol/l) were within the normal range. The maximum inhibition of erythrocyte cholinesterase activity in any individual, relative to their baseline value before dosing, was <20% (Table 3) and the overall patterns were similar in groups receiving placebo or phosmet. The Meeting concluded that there were no consistent, biologically significant effects of treatment with phosmet at doses of 4 mg/kg bw in men and 2 mg/kg bw in women. The NOAEL was 2 mg/kg bw, the highest dose tested in both sexes (Cameron, 1999).

3. Other issues

There are a number of other issues that might impact upon the use of the data from studies in human volunteers for setting an acute reference dose for phosmet.

The study in human volunteers (Cameron, 1999) only measured parameters that could be evaluated by non-invasive techniques, plus blood sampling for haematology, clinical chemistry and cholinesterase activities. It is necessary to confirm that there are no other effects that are critical to the evaluation of phosmet. In two studies there was an indication that brain acetylcholinesterase activity was inhibited to a greater extent than erythrocyte or plasma cholinesterases. However, in the majority of studies the blood cholinesterases were the more sensitive. Overall, the Meeting considered that plasma and erythrocyte cholinesterases are adequate surrogates for nervous system acetylcholinesterase after a single oral exposure to phosmet.

In the study in human volunteers (Cameron, 1999), while doses of up to 4 mg/kg bw were given to men, women were given only a dose of 2 mg/kg bw. As no clear effects were seen at the highest dose in each sex, it is not possible to determine whether there is any difference in the susceptibility of men and women to phosmet. In animal studies, there is again no consistent pattern. In some studies, females showed a greater inhibition than males after moderate doses, whereas in other studies this finding was reversed. At doses approximating to NOAELs, the Meeting considered that there was no consistent evidence relating to a sex difference in response to phosmet.

The 1998 JMPR concluded that: "... there was no evidence that phosmet could produce clinical signs of delayed polyneuropathy or significantly inhibit neuropathy target esterase" (Annex 1, reference 85).

The 1994 JMPR concluded that there was no evidence for carcinogenicity in either rats or mice (Annex 1, reference 73). The 1998 JMPR concluded that "... no further characterization of mutagenicity was required" (Annex 1, reference 85).

In a two-generation study of reproduction in rats given diets containing phosmet (Meyer & Walberg, 1990), reviewed by the 1994 Joint Meeting (Annex 1, reference 73), reductions in mating and fertility were seen at a dietary concentration of ≥ 80 mg/kg. Reductions of >35% (range, 37–59%) in erythrocyte acetylcholinesterase activity were seen in parental animals at 80 mg/kg. Minimal inhibition (about 10%) of erythrocyte acetylcholinesterase activity was seen in parental animals receiving phosmet at a dietary concentration of 20 mg/kg. Pup weight and survival were reduced at 300 mg/kg. The overall NOAEL was 20 mg/kg (equal to 1.3 mg/kg bw per day) on the basis of parental toxicity and

effects on reproductive performance. The Meeting considered that because the reproductive effects were present at doses that produced significant inhibition of acetylcholinesterase activity and after repeated dosing, a single dose that did not produce significant inhibition of acetylcholinesterase activity would not be likely to produce the reproductive effects.

In study of acute neurotoxicity in rats given phosmet by gavage, erythrocyte acetylcholinesterase activity was inhibited by >70% at 22.5 mg/kg bw and by about 10% at 4.5 mg/kg bw. Brain acetylcholinesterase activity was inhibited by >60% at 22.5 mg/kg bw (Cappon, 1998). These results suggest that rabbits given phosmet at a dose of 15 mg/kg bw (as in the study of developmental toxicity by Moxon, 1991) would show significant inhibition of acetylcholinesterase activity.

Comments

In an acceptable¹ double-blind, randomized study, groups of volunteers received a single dose of phosmet (purity, 96%) or placebo, in a capsule, with water. Six subjects receiving phosmet were paired with three subjects receiving placebo, for each dose. Men received a dose of 1, 2 or 4 mg/kg bw, and women received a dose of 2 mg/kg bw. A wide range of investigations, including assays for erythrocyte cholinesterase activity, was performed before and after dosing (up to 168 h). There were no adverse findings at any dose. The pattern of clinical signs, results of investigations and cholinesterase activities were similar in groups receiving test substance and placebo. The Meeting noted that females had only been given a dose of 2 mg/kg bw and concluded that the overall NOAEL for both sexes was thus 2 mg/kg bw.

The Meeting considered the study in volunteers together with other data on the toxicity of phosmet. The Meeting paid particular attention to the data on fetotoxicity from the study of developmental toxicity in rabbits, which had been used to derive the acute RfD in 1998. The skeletal effects (reduced ossification) seen at 5 mg/kg bw per day in this study were not reproduced at a dose of 15 mg/kg bw per day and were mostly within the ranges for contemporary historical controls for the test facility. The forepaw flexure observed in four out of 132 fetuses receiving a dose of 5 mg/kg bw per day was not present in the database for contemporary historical controls, but there was no dose-response relationship, this finding being present in a single fetus out of 118 receiving a dose of 15 mg/kg bw per day. Taking into account the absence of a dose-response relationship and the data on historical controls, the Meeting concluded that there were no clear compound-related effects at a dose of 5 mg/kg bw per day. The altered ossification observed at 15 mg/kg bw per day was seen in the presence of cholinergic signs and significantly reduced maternal body weight gain. The Meeting concluded that the fetal effects were unlikely to occur after a single dose that did not induce significant inhibition of acetylcholinesterase activity.

The Meeting established an acute RfD of 0.2 mg/kg bw based on the NOAEL of 2 mg/kg bw (the highest dose tested) for inhibition of erythrocyte cholinesterase in men and women, and a safety factor of 10.

The Meeting recognized that it was possible that the acute RfD might be refined after a full evaluation of the complete database on phosmet.

¹ Annex 5, reference 83, page 5

Dietary risk assessment

International estimated short-term intake (IESTI) for phosmet was calculated for the raw or processed commodities for which appropriate data on residues and consumption were available. The IESTI for the general population represented 0–90% of the acute RfD. The IESTI for children aged ≤ 6 years represented 0–230% of the acute RfD; the short-term intakes for apples and pears were 150% and 230% of the acute RfD, respectively. The information presented to the Meeting precluded the conclusion that the acute dietary intake for these commodities would be below the acute RfD.

The Meeting concluded that the short-term intake of residues of phosmet from uses that have been considered by the JMPR, with the exception of apples and pears, is unlikely to present a public health concern.

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PYRACLOSTROBIN

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Explanation

Pyraclostrobin is the provisionally approved ISO name for methyl *N*-{2-[1-(4-chlorophenyl)-1*H*-pyrazol-3-yloxymethyl]phenyl}(*N*-methoxy)carbamate (Figure 1). Pyraclostrobin is a member of the strobilurin group of fungicides. The strobilurin fungicides act through inhibition of mitochondrial respiration by blocking electron transfer within the respiratory chain, which in turn causes important cellular biochemical processes to be severely disrupted, and results in cessation of fungal growth. Pyraclostrobin has not been evaluated previously by the JMPR.

The specifications for the active ingredient, pyraclostrobin, permit a maximum content of 0.0003% (3 mg/kg of feed) of the impurity dimethyl sulfate. Dimethyl sulfate is both mutagenic and carcinogenic. For a substantial proportion of the toxicological studies considered in this monograph, there is uncertainty about the presence and level of this impurity in the pyraclostrobin used, although the studies of mutagenicity were performed with material known to contain dimethyl sulfate at 1 mg/kg of feed. This uncertainty will need

Figure 1. Pyraclostrobin and its principle subcomponents

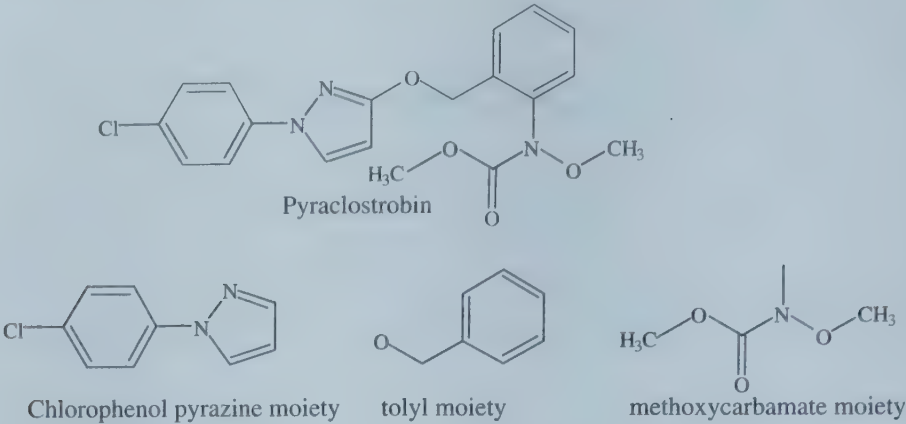


Table 1. Standard clinical chemistry parameters for studies of repeated doses

Haematology	Clinical chemistry	Urine analysis
Differential blood count	Alanine aminotransferase	Bilirubin
Erythrocyte count	Albumin	Blood
Erythrocyte volume fraction	Alkaline phosphatase	Colour
Haemoglobin	Aspartate aminotransferase	Glucose
Leukocyte count	Bilirubin	Ketones
Mean corpuscular haemoglobin (MCH)	Brain cholinesterase	Nitrite
	Calcium	pH
Mean corpuscular haemoglobin concentration (MCHC)	Chloride	Protein
Mean corpuscular volume (MCV)	Cholesterol	Sediment
Platelet count	Creatinine	Specific gravity
Prothrombin time	Erythrocyte cholinesterase (ECHE)	Turbidity
	Globulin	Urobilinogen
	Glucose	Volume
	Inorganic phosphate	
	Magnesium	
	Potassium	
	Protein (total)	
	Serum cholinesterase (SCHE)	
	Serum-γ-glutamyltransferase	
	Sodium	
	Triglycerides	
	Urea	

to be taken into account when performing risk assessments for pyraclostrobin. The available body of analytical information however, suggests that the concentration of DMS in nearly all batches was less than 0.0001%.

Evaluation for acceptable daily intakes

Unless otherwise stated, the studies evaluated in this monograph were certified as having been performed in compliance with good laboratory practice and in accordance with the relevant OECD test guidelines. As these guidelines specify the tissues normally examined and the clinical pathology tests normally performed, only the exceptions to these guidelines are reported here, to avoid repetitive listing of study parameters. For ease of reference, the standard test parameters for studies of repeated doses are provided in Tables 1, 2 and 3.

Table 2. *Organs weighed at sacrifice in studies of repeated doses*

Organs weighed at sacrifice
Adrenal gland(s)
Brain
Epididymis (ides)
Heart
Kidney(s)
Liver
Ovary (ies)
Spleen
Testes
Thymus gland

Table 3. *Tissues examined microscopically in studies of repeated doses*

Tissues examined microscopically			
Adrenal gland(s)	Heart	Pancreas	Spleen
Aorta	Ileum	Parathyroid gland(s)	Sternum (and marrow)
Bone marrow (femur)	Jejunum	Pituitary gland	Stomach
Brain	Kidney(s)	Prostate gland	Testis(es)
Caecum	Liver	Rectum	Thymus gland
Colon	Lung(s)	Salivary gland(s)	Thyroid gland(s)
Duodenum	Lymph nodes	Sciatic nerve(s)	Trachea
Epididymis(ides)	Mammary gland	Seminal vesicles	Urinary bladder
Eye(s)	(females)	Skeletal muscle	Uterus
Femur (with knee	Oesophagus	Skin	Vagina
joint/glenoid surface)	Ovary(ies)	Spinal cord	
	Oviducts		

1. Biochemical aspects

1.1 Absorption distribution and excretion

(a) Oral administration

The absorption, distribution, and elimination of pyraclostrobin were studied in male and female Wistar rats (aged at least 7 weeks) after oral administration of pyraclostrobin (purity, >98%) radiolabelled with ¹⁴C at either the tolyl or chlorophenyl rings.

In a preliminary test, two male and two female rats were assessed for clinical signs for at least 24h after dosing with unlabelled pyraclostrobin at 50mg/kgbw; the dose was well tolerated.

In a series of four experiments, the excretion of pyraclostrobin was studied in excreta collected at 6, 12 and 24h after dosing, and at 24h intervals thereafter for 168h, or until 90% of the applied radioactivity had been excreted. In the first three experiments, groups of four male and four female rats were given a single oral dose of [¹⁴C]tolyl- or [¹⁴C]chlorophenyl-labelled pyraclostrobin or unlabelled pyraclostrobin at 50mg/kgbw. In the fourth experiment, four rats of each sex were given a single oral dose of [¹⁴C]tolyl-labelled pyraclostrobin at 5mg/kgbw. At the end of each of these experiments, the animals were sacrificed and the heart, liver, spleen, bone, skin, lung, ovaries, bone marrow, carcass, muscle, kidney, testes, brain, pancreas, uterus, adipose tissue, stomach and contents, thyroid glands, adrenal glands, blood/plasma and intestinal tract and contents were assessed for

radioactivity. Exhaled air was also collected from two males in each of the two experiments using radiolabelled pyraclostrobin in order to determine exhalation of ¹⁴C-labelled gases.

Two additional experiments were conducted to examine blood concentrations of radioactivity after administration of [¹⁴C]tolyl-labelled pyraclostrobin at 5 or 50 mg/kg bw. Blood samples (100–200 µl) were taken from animals at 0.5, 1, 2, 4, 8, 24, 48, 72, 96 and 120 h after dosing, and the amount of radioactivity in whole blood and plasma was assessed. Tissue distribution was examined in animals sacrificed at 0.5, 8, 20 and 42 h after dosing at 5 mg/kg bw, and at 0.5, 24, 36 and 72 h after dosing at 50 mg/kg bw. The heart, liver, spleen, bone, skin, lung, ovaries, bone marrow, carcass, muscle, kidney, testes, brain, pancreas, uterus, adipose tissue, stomach and contents, thyroid glands, adrenal glands, blood/plasma and intestinal tract and contents were assessed for radioactivity. To examine biliary excretion of pyraclostrobin, bile ducts of the animals were cannulated and bile was collected at 3 h intervals until 48 h after administration of [¹⁴C]tolyl-labelled pyraclostrobin at 5 or 50 mg/kg bw in four animals of each sex at each dose (the duration depended on the health of the animals and the excretion rate at later time-points).

In rats given a single dose of [¹⁴C]tolyl-labelled pyraclostrobin at either 5 or 50 mg/kg bw, plasma concentrations of radioactivity initially peaked after 0.5–1 h; there was a secondary peak after 8 h in males at 5 or 50 mg/kg bw and females given 5 mg/kg bw, and after 24 h in females given 50 mg/kg bw. The magnitude of the difference in the time to peak for females, given the high dose, is likely to be at least partially artifactual owing to the absence of a sampling point between 8 and 24 h. After the second peak, plasma concentrations declined to <0.1 µg equivalent/g after 120 h. The terminal half-lives were similar in males and females, but were 50% longer at 5 mg/kg bw than at 50 mg/kg bw. The area under the curve of plasma concentration–time was approximately proportional to dose for each sex, indicating that absorption was not saturated at the higher dose. Key kinetic data are shown in Table 4.

After a single oral dose of [¹⁴C]tolyl-labelled pyraclostrobin at 50 mg/kg bw, the highest concentrations of radioactivity were found in the gastrointestinal tract (gut, 28–39 µg equivalent/g; gut contents, 63–92 µg equivalent/g; stomach, 325–613 µg equivalent/g; stomach contents, 1273–1696 µg equivalent/g) after 0.5 h. The liver (13–25 µg equivalent/g) had higher concentrations of radioactivity than the kidneys (4–7 µg equivalent/g) and plasma

Table 4. The kinetics of pyraclostrobin in rats

Parameter	Dose (mg/kg bw)			
	5		50	
	Males	Females	Males	Females
First peak blood concentration (µg equivalent/g plasma)	0.432	0.537	1.96	2.62
Time to peak (h)	1.0	0.5	0.5	0.5
Second peak blood concentration (µg equivalent/g plasma)	0.458	0.353	2.04	1.77
Time to peak (h)	8.0	8.0	8.0	24.0
C _{max} (µg/g)	0.458	0.537	2.04	2.62
Initial t _{1/2} (h)	9.0	10.5	—	—
Terminal t _{1/2} (h)	37.4	31.6	20.7	19.7
AUC (µg Eq*h/g)	9.46	8.74	93.97	66.41
Clearance (g/min)	8.81	9.54	8.87	12.4

From Leibold et al. (1998)
AUC, Area under curve

(2–6 µg equivalent/g), with lowest values being recorded in the bone (0.1–0.3 µg equivalent/g) and brain (1–2 µg equivalent/g). After 72 h, tissues and organs contained ≤ 2.6 µg equivalent/g. After a dose of 5 mg/kg bw, the highest concentrations of radioactivity were also found in the gastrointestinal tract (gut, 5 µg equivalent/g; gut contents, 7–9 µg equivalent/g; stomach, 49–89 µg equivalent/g; stomach contents, 160–205 µg equivalent/g) after 0.5 h. After 42 h, tissues and organs contained ≤ 0.7 µg equivalent/g. In rats that were pre-treated with unlabelled pyraclostrobin for 14 days and given a single oral dose of [^{14}C]tolyl-labelled pyraclostrobin at 5 mg/kg bw, the highest concentrations of radioactivity after 120 h were found in the thyroid gland (0.18–0.35 µg equivalent/g) and the liver (0.1 µg equivalent/g). In all other tissues, the concentration of radioactivity recorded was < 0.1 µg equivalent/g. The rapid and essentially complete excretion of pyraclostrobin and the decline of tissue concentrations to low levels over the observation period, suggests a low potential for accumulation.

The overall recovery of radioactivity was 91–105% in all experiments. In the first 48 h after a single oral dose of [^{14}C]tolyl-labelled pyraclostrobin at 5 or 50 mg/kg bw, 10–13% of the administered radioactivity was excreted in the urine and 74–91% was excreted in the faeces. The total amount of radioactivity excreted in the urine and faeces after 120 h was 11–15% and 81–92%, respectively. A similar pattern of excretion was observed in rats that were pre-treated with unlabelled pyraclostrobin for 14 days and given a single oral dose of [^{14}C]tolyl-labelled pyraclostrobin at 5 mg/kg bw of (12–13% in the urine and 76–77% in the faeces after 48 h; 12–14% in the urine and 79–81% in the faeces after 120 h) and in rats given a single oral dose of chlorophenyl-labelled pyraclostrobin at 50 mg/kg bw (11–15% in the urine and 68–85% in the faeces after 48 h; 12–16% in the urine and 74–89% in the faeces after 120 h). There was no detectable radioactivity in the expired air from rats treated with [^{14}C]tolyl- or [^{14}C]chlorophenyl-labelled pyraclostrobin at 50 mg/kg bw. In tissues and organs, the radioactivity that remained after 120 h was < 1 µg equivalent/g at 50 mg/kg bw and < 0.1 µg equivalent/g at 5 mg/kg bw. Within 48 h after administration of [^{14}C]tolyl-labelled pyraclostrobin at 5 or 50 mg/kg bw of, 35–38% of the administered radioactivity was excreted via the bile, indicating, in conjunction with observations on urinary excretion, that approximately 50% of the administered dose had been absorbed (Leibold et al., 1998).

(b) *Dermal application*

The absorption and, to a limited extent, the distribution and excretion of ^{14}C -labelled pyraclostrobin (in Solvesso) in groups of 16 male Wistar rats was assessed after a single dermal application at a nominal dose of 0.015, 0.075 or 0.375 mg/cm², corresponding to 0.15, 0.75 and 3.75 mg/animal or approximately 0.8, 4 and 18 mg/kg bw. Animals were exposed to the test material for 4 (four rats per group) or 8 (12 rats per group) h and four rats per group were sacrificed at 4, 8, 24 or 72 h after the start of the exposure. An area of approximately 10 cm² on the shoulders was clipped free of hair and was washed with acetone 24 h before dosing. A silicone ring was glued to the skin and the test substance preparation (10 µl/cm²) was administered with a syringe, which was weighed before and after application. A nylon mesh was then glued to the surface of the silicone ring and covered with a porous bandage. After the exposure period, the protective covers were removed and the exposed skin was washed with a soap solution. After sacrifice, the concentration of radioactivity in the excreta, blood cells, plasma, liver, kidneys, carcass, treated and untreated skin was assessed. Radioactivity in the cage and skin wash and the protective covering, including the silicone ring, was also assessed. In all groups, 99–110% of the radioactivity was

Table 5. Dermal absorption of pyraclostrobin in rats (mean recovery of radioactivity (%))

Recovery	Exposure (h)	Sacrifice (h)	Dose (mg/cm ²)		
			0.375	0.075	0.015
Absorbed*	4	4	0.51	0.43	0.55
	8	8	0.51	0.85	0.64
	8	24	1.19	2.56	1.49
	8	72	1.58	2.59	1.57
Skin (application site)	4	4	7.11	7.56	7.78
	8	8	7.85	10.85	10.60
	8	24	9.25	12.61	6.40
	8	72	3.37	13.68	12.10
Urine/faeces	4	4	0.01/0.01	0.01/0.01	0.01/0.00
	8	8	0.04/0.01	0.05/0.02	0.03/0.01
	8	24	0.16/0.42	0.22/0.56	0.17/0.49
	8	72	0.22/0.91	0.38/1.76	0.27/1.04
Total recovery	4	4	107	102	103
	8	8	99	105	109
	8	24	100	110	105
	8	72	105	104	100

From Leibold & Hoffmann (1999)

*Radioactivity recovered in excreta, cage wash, blood, kidney, liver and the carcass

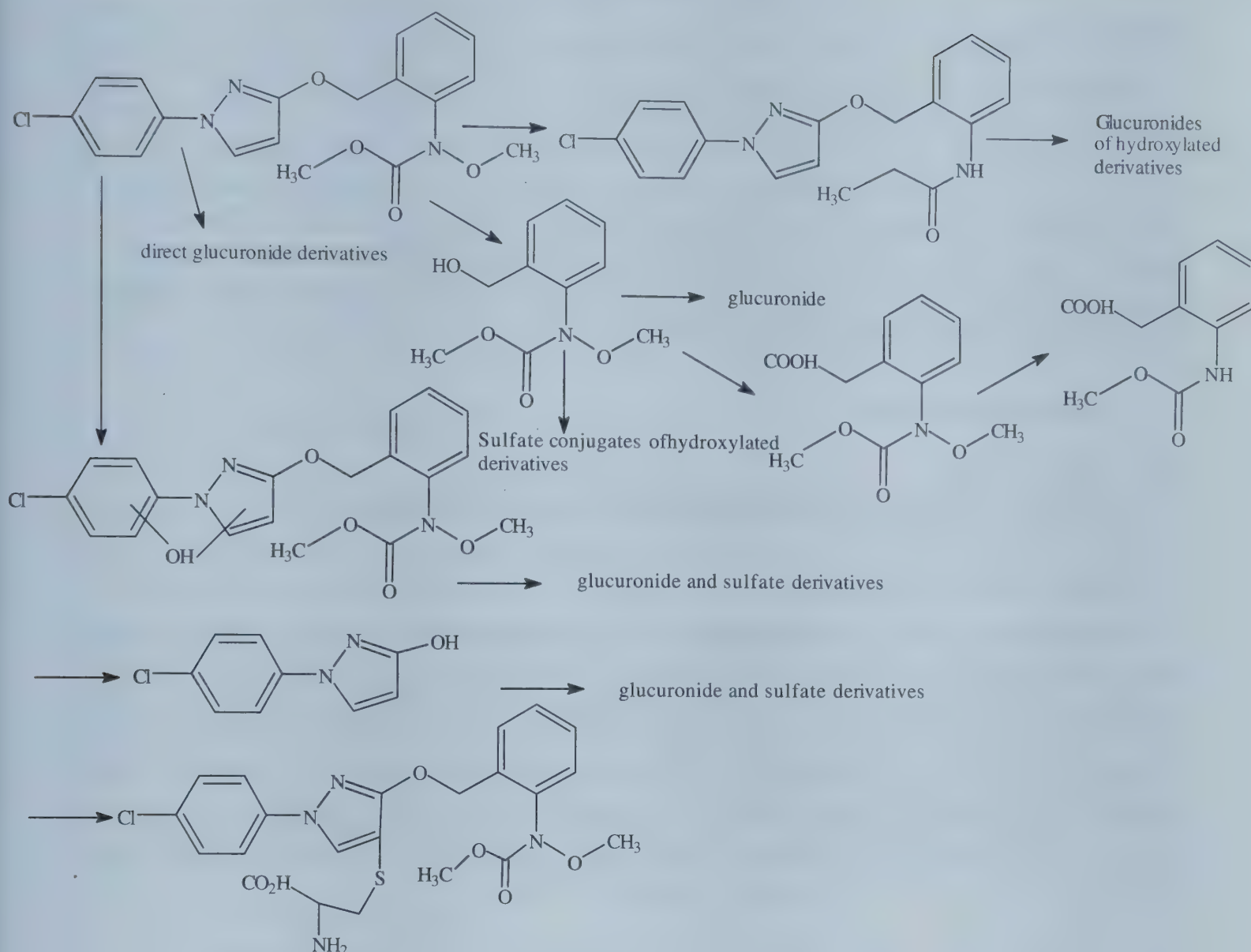
recovered. At sacrifice at 72 h, after an 8 h exposure, 1.6–2.6% of the administered dose was absorbed, 22–26% was on the skin or in the skin wash, and 72–80% was recovered on the protective cover. Only 0.2–0.4% and 0.9–1.8% was excreted in the urine and faeces, respectively (Leibold & Hoffmann, 1999).

In a second study of dermal application, ¹⁴C-labelled pyraclostrobin (in a commercial formulation, details of which were not provided) was applied at a dose of 15, 75 and 375 µg/active ingredient/cm² to the upper surface of epidermal membranes from Wistar rats and human cadavers in vitro, and left unoccluded for 24 h. Skin samples were obtained from the dorsal/dorso-lumbar region from sacrificed rats and human cadavers and were mounted in glass diffusion cells to give a surface area of approximately 1.77 cm². The receptor chamber contained an ethanol/water (1 : 1) mix as the receptor fluid. Ten skin preparations per species and dose were assessed. Only tissues in which the epidermal layer was intact were used in the study. On the day before application of the formulation, the integrity of the skin was assessed by measuring the penetration of tritiated water, which was applied to the epidermal surface. The test material was applied to the upper surface of the epidermal membranes and duplicate aliquots (100 µl) of receptor fluid were taken at 1, 2, 4, 6, 10 and 24 h subsequently. Residual test material was washed from the skin surface with a 10% w/v soap solution, and the washings, remaining receptor fluid, ethanol washings of the dismantled diffusion cells and solubilized skin membranes were retained for analysis of residual radioactivity. During the 24 h after application, 21–51% and 3–8% of the applied [¹⁴C]pyraclostrobin was absorbed across the rat and human epidermis respectively, with 13–22% (rat) and 14–17% (human) of the dose recovered on the skin. The majority of absorption by rat skin occurred in the first 6 h, whereas total absorption by human skin increased throughout the entire 24 h period. In total, 91–95% (rat) and 88–106% (human) of the applied dose was recovered (Thomley & Wood, 1999).

1.2 Biotransformation

Tissues, excreta and bile from animals used in the toxicokinetics studies and from additional groups given a single dose at 50 mg/kg bw per day (to provide more material for

Figure 2. Proposed metabolic pathways for pyraclostrobin in rats



From Velic (1999)

analysis) were analysed for metabolites of pyraclostrobin. In order to determine the metabolites in the plasma, liver and kidneys, additional groups were treated with a single dose of [^{14}C]tolyl- or [^{14}C]chlorophenol ring-labelled pyraclostrobin at 5 and 50 mg/kg bw and sacrificed 8 h later. Metabolites were identified using high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR). The metabolism of pyraclostrobin proceeded through three main pathways primarily involving alterations to the three major portions of the pyraclostrobin molecule.

The methoxy group on the tolyl-methoxycarbamate moiety was readily lost, with few major metabolites retaining this group. Hydroxylation of the aromatic and/or pyrazole rings was followed by glucuronide and occasionally sulfate conjugation, and many metabolites were derived from the chlorophenol-pyrazole or tolyl-methoxycarbamate moieties of pyraclostrobin, following cleavage of the ether linkage, with subsequent ring hydroxylation and glucuronide or sulfate conjugation. Metabolites were similar in both sexes and at all doses. No unchanged parent compound was found in the bile or urine and only small amounts in the faeces. Compounds dominating the identified metabolites recovered from the urine were: ring-hydroxylated pyraclostrobin; the chlorophenol pyrazole moiety hydroxylated on the

pyrazole ring with or without a sulfate conjugate; a glucuronide of the tolyl-methoxycarbamate moiety; and a benzoic acid derivative of the tolyl-methoxycarbamate moiety. In the faeces, the dominant metabolite was a demethoxylated and pyrazole ring hydroxylated pyraclostrobin. In the bile, the primary metabolite was a glucuronide of pyraclostrobin hydroxylated on the pyrazole ring at the 4' position and this compound, together with the demethoxylated derivative found in the faeces, was also the dominant metabolite isolated from the plasma and the liver. Demethoxylation of the methoxycarbamate moiety appeared to occur primarily in the gut, as the major metabolite in the bile retains this group intact whereas in the faeces the major metabolite is the demethoxylated derivative. Most of the radiolabel isolated from the kidneys was in the form of the unchanged parent compound and a demethoxylated derivative (Velic, 1999).

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity of pyraclostrobin is summarized in Table 6.

(a) Oral administration

Clinical signs after oral administration of pyraclostrobin consisted of dyspnoea, staggering, piloerection, and diarrhoea in all animals, resolving by day 6. There were no pathology findings. In a study of acute inhalation using acetone as the solvent, all animals at 1.070 and 5.300 mg/l died on the day of exposure. At 0.310 mg/l, bloody discharge from the nose (two males), piloerection and smeared fur (10 out of 10 animals) were observed. All effects had resolved in surviving animals by day 7. Where Solvesso was used as the solvent, all males and four out of five females at 7.3 mg/l died, and one out of 10 animals died at each of the two lower doses. There were no deaths at 0.89 mg/l.

Table 6. Studies of acute toxicity with pyraclostrobin

Species	Strain	Sex	Route and vehicle	Dose (mg/kg bw)	Purity (%)	LD ₅₀ (mg/kg bw) or LC ₅₀ (mg/l air)	Reference
Rat	Wistar	Male & female	Oral ^a	2000, 5000 mg/kg bw, in 0.5% aqueous Tylose CB 30000	≥98.2	>5000 mg/kg bw (no deaths)	Wiemann & Hellwig (1998a)
Rat	Wistar	Male & female (five of each sex)	Dermal ^b	2000 mg/kg bw in 0.5% aqueous Tylose CB 30000	≥98.2	>2000 mg/kg bw (no deaths, no pathology, slight erythema resolving within 2 days)	Wiemann & Hellwig (1998b)
Rat	Wistar	Male & female (five of each sex)	Inhalation ^c	0, 0.89, 1.96, 4.07, 7.3 mg/l, 40% in Solvesso (head and nose only), 4 h	≥98.2	>4.07 mg/l, <7.3 mg/l	Gamer et al. (2001)
Rat	Wistar	Male & female (five of each sex)	Inhalation ^c	0, 0.310, 1.070, 5.270 mg/l, in acetone 1:2 (head and nose only), 4 h	≥98.2%	>0.310 mg/l, <1.070 mg/l	Gamer & Hoffmann (1997)

^a Dose volume = 10 and 20 ml/kg bw

^b Intact skin, 24 h exposure, 50 cm² area

^c As pyraclostrobin is a viscous fluid with a "negligibly low" vapour pressure (2.6×10^{-10} hPa), it was dissolved in acetone (4-h LC₅₀, approximately 80 mg/l) or Solvesso to facilitate aerosolization. Mean mass aerodynamic diameter was 1.0, 1.2 and 2.9 µm for the 0.310, 1.070 and 5.270 mg/l groups, respectively, for the study using acetone as the solvent, and between 2.7 and 4.3 µm for the study using Solvesso as the solvent

(b) *Dermal irritation*

Undiluted pyraclostrobin (500 mg, purity 98.2%) was applied to the shaved, intact skin on the back flanks of six New Zealand White rabbits under a semi occlusive bandage for 4 h. At the end of the exposure period, the test substance was removed and the treated area was rinsed with polyethylene glycol and water. There were no mortalities. Erythema was observed in all animals from 1 h after removal of the bandage and persisting in most animals until day 8, and in three animals until day 15. The maximum Draize score for erythema was 3 and the average scores at day 1 and 8 were 2 and 1.5 respectively. Oedema with a Draize score of 1 was observed in four out of six rabbits on day 1, resolving in all except two rabbits by day 8, but persisting in one rabbit until day 15. It was concluded that pyraclostrobin is a slight but prolonged skin irritant (Wiemann & Hellwig, 1998c).

(c) *Ocular irritation*

Pyraclostrobin (0.1 ml; purity, 98.2%) was instilled into the conjunctival sac of the right eye of one male and five female New Zealand white rabbits. After 24 h, the test material was washed out with tap water. The left eye was not treated and served as a control. There were no deaths during the study. Conjunctival redness (score = 1–3) was observed in all animals up to 3 days after treatment, with swelling observed in five out of six rabbits at 1 h (score = 1), six out of six rabbits on day 1 (average score = 1.2), three out of six rabbits on day 2 (score = 1), and two out of six rabbits on day 3 (score = 1). Discharge (score = 1) occurred in one out of six rabbits at 1 h. There were no corneal or iridal effects and all conjunctival effects had resolved by day 8. "Loss of hair at the margins of the eyelids" occurred in six out of six rabbits from 1 day after treatment. Under the conditions of the study, pyraclostrobin was a slight ocular irritant in rabbits (Wiemann & Hellwig, 1998d).

(d) *Skin sensitization*

In a Magnusson-Kligman maximization test, intradermal injections (2×0.1 ml) of Freund adjuvant in a 0.9% aqueous solution of sodium chloride (1:1), 5% pyraclostrobin in Freund adjuvant and 5% pyraclostrobin in 1% Tylose CB 30000 in Aqua bidest (Tylose) were given to the left and right shoulders of each of 20 guinea-pigs. Sites were evaluated 24 h after injections were given. One week later, 5% pyraclostrobin in Tylose (1 ml) was applied to a gauze patch of surface area 2×4 cm and administered topically to the same sites, then covered with an occlusive dressing for 48 h, after which time the sites were assessed. On day 22, all animals were challenged with 0.5 ml of 1% pyraclostrobin in Tylose (right flank) and Tylose alone (left flank). A second challenge was performed on day 29, when the test substance was applied to the left flank and the vehicle applied to the right flank. All challenge sites were evaluated 24 and 48 h after removal of the occlusive dressings. There were no deaths and all animals gained body weight normally over the study. Although intradermal injections of Freund adjuvant, 5% pyraclostrobin in Freund adjuvant and 5% pyraclostrobin in Tylose caused moderate and confluent erythema (Draize score = 2) and swelling in all animals, as did an occluded topical application of 5% pyraclostrobin in Tylose, the first and second challenges with 1% pyraclostrobin in Tylose and Tylose alone caused no effect in any animal at 24 or 48 h. The sensitivity of the procedure was confirmed in an assay with the positive controls technical-grade alpha-hexyl cinnamaldehyde technical (85%) and Lutrol E 400 DAB (Lutrol). Pyraclostrobin was not a skin sensitizer in guinea-pigs in this study (Wiemann & Hellwig, 1998e).

2.2 *Short-term studies of toxicity*

Mice

Groups of five male and five female B6C3F₁ mice were given pyraclostrobin (in 0.5% aqueous carboxymethylcellulose) at a dose of 0 or 4 mg/kg bw per day for 1 week by gavage. Mice were also given diets containing pyraclostrobin at a concentration of 0 or 18 mg/kg of feed for males and 0 or 15 mg/kg of feed for females, for 1 week. Food consumption and body weight were determined daily and animals were examined for mortality and clinical signs of toxicity at least once per day. At the end of the experiment, animals were sacrificed without further examination. Mean intakes of pyraclostrobin were 5.5 mg/kg bw per day in males at 18 mg/kg, and 7.2 mg/kg bw per day in females at 15 mg/kg. After 1 week, food consumption was 31% lower than that of controls in females at 15 mg/kg, but there was no treatment-related effect on body-weight gain and there were no other effects of treatment in any of the treated groups of mice. Data for individual animals were not supplied. This was a supplemental study initiated to address the appropriateness of using an apparently lower body-weight gain in treated animals in a study of developmental toxicity in rabbits as an end-point on which to base the establishment of an acute reference dose (RfD). In conjunction with similar studies in rabbits and rats, this study was intended to demonstrate the species-specific variability in food intake and body-weight gains in rabbits. Because of the limited parameters examined in this study, it was not adequate for the purposes of risk assessment and a no-observed-adverse-effect level (NOAEL) could not be established (Mellert, 2002a).

Groups of 10 male and 10 female B6C3F₁ mice (aged 47–49 days) were given diets containing pyraclostrobin (purity, 98.5%) at a concentration of 0, 50, 150, 500, 1000, or 1500 mg/kg of feed (equal to 0, 9.2, 30, 120, 270 and 480 mg/kg bw per day) for 3 months. Mice were checked at least once daily for mortality and signs of toxicity, and a comprehensive clinical examination was performed once per week. Body weight and food consumption were recorded once weekly and water consumption was assessed daily. Blood was collected from fasted animals and haematology (excluding prothrombin time) and clinical chemistry (excluding brain, erythrocyte and serum cholinesterases) parameters were assessed in all animals. After 3 months of treatment, all mice were fasted, sacrificed and necropsied. All animals were examined grossly. In the control group and in the group receiving pyraclostrobin at 1500 mg/kg, tissues (including gall bladder) were examined microscopically. The thymus gland, lungs, liver, kidneys, adrenal glands (females), stomach, duodenum, jejunum, ileum and mesenteric lymph nodes were examined microscopically in animals at 50, 150, 500 and 1000 mg/kg, and gross lesions were assessed in all animals affected per group. Organ weights (excluding epididymides, heart and thymus gland) were recorded.

There were no treatment-related clinical signs. Body-weight gain was reduced throughout the study period in males at all doses and in females at ≥ 500 mg/kg of feed in a clear dose-related manner, with males at the highest dose experiencing a slight loss in body weight. Reduced weight gains in females at 50 and 150 mg/kg were slight, not statistically significant except at 150 mg/kg on day 77, and were not apparent before day 28 of treatment. Nonetheless a dose-related trend was apparent in females at all doses from day 28 onwards. Although spillage of food hindered interpretation, consistently lower food conversion efficiency values at 1000 and 1500 mg/kg suggested a relationship to treatment. No changes in water consumption between the groups were noted. Reductions were seen at 1500 mg/kg in haemoglobin concentration, MCV and MCH in both sexes, and in erythro-

cyte volume fraction in males. Platelet counts were increased in both sexes at 1500 mg/kg and in males at 500 and 1000 mg/kg. Haemoglobin concentration was also reduced in females at 1000 mg/kg and erythrocyte volume fraction was reduced in males at 150 mg/kg and above. Leukopenia was seen in both sexes at 1000 and 1500 mg/kg and in females at 500 mg/kg and possibly also 150 mg/kg. There was a reduction in the concentration of eosinophils in males at 50 mg/kg and above, in lymphocytes in both sexes at 1000 and 1500 mg/kg and females at 500 mg/kg, and in monocytes in males at ≥ 500 mg/kg. As individual control animals had eosinophil counts ranging from 0 to $0.43 \times 10^9/l$ the apparent, slight, effect at 50 mg/kg was not considered to be toxicologically significant. Increases were seen in serum cholesterol in females at 1500 mg/kg and urea concentration in both sexes at ≥ 150 mg/kg. The values for urea concentration in males at the lowest dose were within the range for historical controls, but in view of the clear increase at ≥ 150 mg/kg, a substance-related effect at 50 mg/kg could not be ruled out. Decreases were observed in total protein in both sexes at 1500 mg/kg and in females at 1000 mg/kg, in globulin concentration in both sexes at 1000 and 1500 mg/kg, and in triglyceride concentration in both sexes at ≥ 150 mg/kg and in females at 50 mg/kg. Although the reduced concentration of triglyceride in females was not statistically significant a clear dose-response relationship was apparent and examination of values for individual animal confirmed a consistent pattern of reduced values.

A number of organ weight differences between groups were observed which, in the absence of histological alterations in those organs, are likely to be secondary to reduced weight gains and food conversion efficiency in groups receiving pyraclostrobin at >150 mg/kg of feed. Increased relative liver and spleen weights in males at ≥ 500 mg/kg could not be readily attributed to altered weight gains, as the relative (to body weight) liver weight tends to remain stable or decline when weight gain is reduced through reduced food intake or reduced food conversion efficiency, and the increased relative spleen weight correlated with the anaemia and leukopenia observed at 1000 and 1500 mg/kg.

Increased incidences and/or severity was seen in the following findings: thickening of the duodenal mucosa in both sexes at ≥ 500 mg/kg; erosions or ulcers in the glandular stomach in both sexes at ≥ 500 mg/kg and in females at 150 mg/kg; atrophy of the thymus gland in both sexes at ≥ 500 mg/kg and in females at 150 mg/kg; apoptotic bodies in follicles of the mesenteric lymph node in both sexes at 1500 mg/kg and in females at 500 and 1000 mg/kg. Decreases were seen in the incidences of vacuolation in cells of the X-zone in the adrenal cortex in females at ≥ 150 mg/kg and in males at 1500 mg/kg, lipid vacuoles in the kidneys of males at ≥ 500 mg/kg, and fatty infiltration in the liver of both sexes at 1500 mg/kg. A NOAEL was not identified owing to decreased body-weight gains and altered clinical pathology parameters at all doses. (Mellert et al., 1998; Mellert et al., 1999j). Taking into consideration the study of carcinogenicity in mice (doses: 0, 10, 30, 120 mg/kg) the NOAEL for reduced weight gain at 91 days in mice was 30 mg/kg (4 mg/kg bw per day). As the study of carcinogenicity did not examine clinical chemistry parameters, a specific NOAEL for elevated blood urea concentrations could not be identified for this species. Nonetheless, consideration of the dose-response trend in the 3-month study, the observation that the value for this parameter at 50 mg/kg was within the range for historical controls and the absence of abnormal histology and gross pathology in males at 120 mg/kg (17 mg/kg bw per day) and in females at 180 mg/kg (33 mg/kg bw per day) in the study of carcinogenicity in mice suggests that the overall NOAEL in mice of 30 mg/kg (4 mg/kg bw per day) was appropriate.

Table 7. Haematology and clinical chemistry values in mice given diets containing pyraclostrobin for 3 months

Parameter	Dietary concentration (mg/kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
<i>Haematology</i>												
Haemoglobin (mmol/l)	11.8	11.4	11.6	11.5	11.6	11.2	11.4	11.0	11.4	10.9**	10.6***	10.4**
Erythrocyte volume fraction (l/l)	0.57	0.52	0.56	0.53	0.55*	0.52	0.54**	0.52	0.54**	0.51	0.52**	0.50
MCV (10–15l)	48.3	46.4	48.0	46.7	47.3	46.3	47.0*	46.8	46.7**	46.0	42.6***	42.6
MCH (10–15 mol/l)	0.99	1.02	0.99	1.01	1.00	1.00	0.99	1.00*	0.98*	0.98**	0.87***	0.90***
MCHC (mmol/l)	20.6	22.0	20.7	21.6	21.1*	21.7	21.1	21.3***	21.0	21.2***	20.5	21.0***
Platelets (×10 ⁹ /l)	1120	1048	1168	1078	1199	1014	1271**	1086	1205‡	1112	1247**	1236**
White blood cells (×10 ⁹ /l)	5.9	6.0	5.6	5.2	5.2	4.1	6.4	3.7	2.7***	3.3	2.7***	3.2
Eosinophils (×10 ⁹ /l)	0.14	0.04	0.08	0.05	0.06	0.02	0.02	0.00	0.00	0.00	0.00	0.01
Lymphocytes (×10 ⁹ /l)	4.0	3.7	4.2	3.6	3.8	2.9	3.9	2.3	1.5	2.2	0.9	2.0
Monocyte (×10 ⁹ /l)	0.39	0.14	0.23	0.14	0.22	0.11	0.07	0.04	0.01	0.04	0.01	0.09
<i>Clinical chemistry</i>												
Urea (mmol/l)	7.3	6.1	7.9*	6.7	8.8***	9.1**	10.6***	11.1***	12.0***	10.9***	12.0***	9.9***
Total protein (g/l)	63.9	60.0	67.6**	62.2	66.7**	61.7	64.9	59.7	61.4*	55.7***	57.0***	55.2**
Globulin (g/l)	26.0	22.0	27.5**	22.8	27.3	22.4	26.2	20.6**	23.3***	18.7***	21.0***	18.5***
Triglyceride (mmol/l)	1.70	1.53	1.64	1.22	1.15**	0.96*	0.78***	0.58***	0.59***	0.59***	0.47***	0.58***
Cholesterol (mmol/l)	3.5	2.5	4.0*	2.9**	4.0**	2.8	3.7	3.0**	3.8	3.2***	3.3	3.8***

From Mellert et al. (1998) and Mellert et al. (1999j)
M, Male; F, Female; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration
* $p \leq 0.05$; ** $p \leq 0.02$; *** $p \leq 0.002$, ‡ An aberrant value of 528 was excluded from the mean to give a mean of 1205 instead of 1137

Table 8. Organ weights in mice given diets containing pyraclostrobin for 3 months

	Dietary concentration (mg/kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
<i>Organ weights</i>												
<i>Absolute weights</i>												
Body (g)	31.1	22.6	28.6	21.8	26.6**	21.0	23.4**	18.8**	21.1**	17.3**	18.9**	16.2**
Adrenals (mg)	5.5	11.6	5.5	10.8	5.7	10.1*	6.1	8.1**	5.7	6.5**	6.5	6.7**
Brain (mg)	481	494	480	482	477	490	479	481	475	466**	457**	452**
Kidneys (mg)	488	346	432	332	446*	324*	405**	300**	344**	274**	308**	255**
Liver (mg)	1137	1112	1053	1011	1052	988	995**	840**	948**	848**	869**	832**
Spleen (mg)	63.4	73.9	61.0	68.8	59.4	67.2	56.9	60.8*	50.7**	53.5**	44.0**	50.2**
Testes (mg)	232	—	224	—	217*	—	227	—	218	—	203**	—
<i>Relative (to body) weights</i>												
Adrenals	0.018	0.051	0.020	0.050	0.022	0.049	0.026**	0.043*	0.027**	0.037**	0.034**	0.041*
Brain	1.57	2.22	1.71	2.23	1.80**	2.36	2.06**	2.56**	2.26**	2.70**	2.43**	2.80**
Kidneys	1.58	1.55	1.52	1.53	1.68	1.56	1.73	1.60	1.63	1.58	1.63	1.58
Liver	3.68	4.96	3.70	4.65	3.97*	4.70	4.25**	4.45	4.50**	4.89	4.60**	5.14
Spleen	0.21	0.33	0.21	0.32	0.22*	0.32	0.24**	0.32	0.24*	0.31	0.23	0.31
Testes	0.75	—	0.79	—	0.82	—	0.97**	—	1.03**	—	1.08**	—

From Mellert et al. (1998) and Mellert et al. (1999j)
Relative organ weight = organ weight (g)/body weight (g) × 100
* $p \leq 0.05$; ** $p \leq 0.01$

Table 9. Pathology findings in mice given diets containing pyraclostrobin for 3 months

Finding	Dietary concentration (mg/kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
No. of animals examined	10	10	10	10	10	10	10	10	10	10	10	10
<i>Gross findings</i>												
Thickening of duodenum wall	0	0	0	0	0	0	8	6	10	10	10	9
Erosion/ulcer of the glandular stomach	1	2	0	2	1	4	2	7	2	4	4	1
<i>Microscopic findings</i>												
Decreased vacuolation in adrenal cortex X-zone cells												
Grade 1, 2 or 3	0	1	0	1	0	1	1	3	1	0	9	0
Grade 4 or 5	0	0	0	0	0	4	0	7	0	10	0	9
Total	0	1	0	1	0	5	1	10	1	10	9	9
Thickening of duodenum mucosa												
Grade 2	0	0	0	0	0	0	6	6	1	10	0	7
Grade 3	0	0	0	0	0	0	4	0	9	0	10	2
Total	0	0	0	0	0	0	10	6	10	10	10	9
Mean thickness of mucosa (mm)	0.33	0.27	0.32	0.29	0.36	0.32*	0.49**	0.43**	0.48**	0.46**	0.46**	0.44**
Glandular stomach erosion/ulcer	1	1	1	3	2	5	4	7	5	6	8	6
Kidneys, lipid vacuoles	10	10	10	10	10	9	2	7	1	7	0	7
Liver, diffuse fatty infiltration												
Grade 2 or 3	2	3	2	3	3	2	8	3	7	7	4	4
Grade 4	8	7	8	7	7	6	2	3	3	2	0	3
Total	10	10	10	10	10	8	10	6	10	9	4	7
Mesenteric lymph node apoptosis												
Grade 1	0	0	0	0	0	0	1	0	1	5	0	5
Grade 2	0	0	0	0	0	2	0	4	0	1	9	2
Total	0	0	0	0	0	2	1	4	1	6	9	7
Thymus gland atrophy												
Grade 2	0	0	0	0	0	3	2	2	4	3	1	0
Grade 3	0	0	0	0	0	2	1	5	1	4	2	2
Grade 4	0	0	0	0	0	1	0	0	1	1	5	2
Total	0	0	0	0	0	6	3	7	6	8	8	4

From Mellert et al. (1998) and Mellert et al. (1999j)
Grade 1 = minimal in severity/very few in number/very small in size; grade 2 = slight in severity/few in number/small in size; grade 3 = moderate in severity and size/moderate to several in number, grade 4 = severe

Rats

Groups of five male and five female Wistar rats were given pyraclostrobin (in 0.5% aqueous carboxymethylcellulose) at a dose of 0 or 4 mg/kg bw per day by gavage for 1 week, or diets containing pyraclostrobin at a concentration of 0 or 34 mg/kg for 1 week. Food consumption and body weight were determined daily and animals were examined for mortality and clinical signs of toxicity at least once per day. At the end of the experiment, animals were sacrificed without further examination. The dietary concentration of 34 mg/kg of feed corresponded to mean intakes of pyraclostrobin of 3.5 mg/kg bw per day in males and 3.8 mg/kg bw per day in females. Lower body-weight gain (up to 33%) was observed in females receiving diet containing pyraclostrobin at 34 mg/kg, but not in females given an equivalent dose of pyraclostrobin by gavage, nor in males given pyraclostrobin either in the diet or by gavage. There were no deaths and no clinical signs of toxicity in any group. Data for individual animals was not supplied. This was a supplemental study initiated to address the appropriateness of using an apparently lower body-weight gain in treated animals in a study of developmental toxicity in rabbits as an end-point on which to base the establishment of an acute RfD. In conjunction with similar studies in rabbits and mice, this study was intended to demonstrate the species-specific variability in food intake and body-weight

gains in rabbits. Because of the limited parameters examined in this study, it was not adequate for the purposes of risk assessment and a NOAEL could not be identified (Mellert, 2002b).

Groups of five male and five female Wistar rats (aged 42 days) were given diets containing pyraclostrobin (purity, 94–99%) at a concentration of 0, 20, 100, 500, or 1500 mg kg of feed (equal to 0, 1.8, 9, 42 and 120 mg kg bw per day) for 4 weeks. Dose selection was based on the results of a preliminary study (BASF Aktiengesellschaft Project No. 24S0376-96061) in which groups of five male and five female Wistar rats were given diets containing pyraclostrobin at a concentration of 400, 3000 or 15000 mg kg of feed for 2 weeks. A separate report was not provided for this study. Rats were checked at least daily for mortality and signs of toxicity, a comprehensive clinical examination was performed weekly, body weight and food consumption were recorded weekly and water consumption was recorded daily. Haematology, urine analysis and clinical chemistry parameters were assessed in all animals, as were gross pathology and organ weights. Histological examination was performed on the liver, spleen, fore-stomach, glandular stomach, duodenum, jejunum, ileum, caecum, colon and rectum of all animals, heart, kidneys, adrenal glands and testes in the control group and at 15000 mg kg only, and gross lesions were assessed in all animals affected.

In the preliminary study, all animals at 15000 mg kg were sacrificed because of excessive toxicity (no other details provided) and at 3000 mg kg, body-weight gain and food consumption were reduced, with signs of anaemia apparent at 400 and 3000 mg kg (no other details provided).

In the main study, there were no deaths or treatment-related clinical signs, but reductions of up to 16% in food consumption at 500 mg kg and of up to 44% at 1500 mg kg were observed. Concomitantly, body-weight gain was reduced by 14 to 32% over the study in both sexes at 1500 mg kg and in males at 500 mg kg, primarily owing to a pronounced reduction of 51–67% at 1500 mg kg during the first week of the study. A slight anaemia characterized by reduced erythrocyte numbers and haemoglobin concentration was observed in females at 500 and 1500 mg kg, with a slight, not statistically significant, reduction in haemoglobin concentration also seen in males at 1500 mg kg. The anaemia correlated with evidence of extramedullary haematopoiesis in the liver and spleen and with increased relative spleen weights in both sexes at 500 and 1500 mg kg. Slight decreases were seen in alanine aminotransferase in both sexes at 500 and 1500 mg kg, and in serum cholinesterase in females at 1500 mg kg. As decreased alanine aminotransferase activity was observed in the 3-month and long-term studies in rats also, this effect is likely to be treatment-related but, as the magnitude of the effect was small and a decrease is not normally associated with adverse organ or system effects, is unlikely to be toxicologically relevant. This conclusion was further supported by studies indicating that alanine aminotransferase (and alkaline phosphatase) activities can be affected by dietary status, as discussed later in this monograph. Urine volume was increased and specific gravity was decreased in both sexes at 1500 mg kg.

Absolute kidney, adrenal and thymus weights were decreased and relative brain weights were increased secondary to reduced body-weight gains at 1500 mg kg. Histology did not reveal any alterations in these tissues. An increased relative liver weight correlated with increased hepatocellular hypertrophy in males at 1500 mg kg, diminished fat storage at 500 and 1500 mg kg, and altered clinical chemistry values at 1500 mg kg (increased total

Table 10. Clinical chemistry findings in rats given diets containing pyraclostrobin for 4 weeks

Parameter	Dietary concentration (mg/kg of feed)									
	0 (control)		20		100		500		1500	
	M	F	M	F	M	F	M	F	M	F
Erythrocyte count ($10^{12}/l$)	8.5	8.1	8.1	7.9	8.1	8.1	8.2	7.6**	8.2	7.4**
Haemoglobin (mmol/l)	9.6	9.5	9.4	9.3	9.6	9.4	9.3	8.8**	8.9	8.8**
MCV (10–151)	53.7	54	54.3	54.7	55.1	54.1	53.9	54.2	53.1	57.5**
MCHC (mmol/l)	21.2	21.6	21.2	21.5	21.3	21.5	20.9	21.5	20.4*	20.9**
Prothrombin time (s)	28	24.9	28.2	24.6	26.9	24.7	28.9**	25.7	30.2*	27.8**
Platelets ($\times 10^9/l$)	769	753	752	814	785	742	780	768	849	861
ALT ($\mu\text{kat}/l$)	1.06	1.03	1.02	0.95	0.91	0.94	0.75**	0.88	0.87	0.78
Total bilirubin ($\mu\text{mol}/l$)	2.9	3.1	2.6	2.6	2.6	2.4	3.3	3.5	5.9**	3.6
Globulin (g/l)	28.2	26.7	27.3	25.9	28.4	26.3	26.5	24.6	24.4	23.1
Glucose (mmol/l)	8.2	8.5	8.5	8.5	8.3	7.8	7.5	7.9	7.3	6.7**
Inorganic phosphate (mmol/l)	3.0	2.6	3.1	2.5	3.0	2.6	2.7	2.4	2.5*	2.4
Serum cholinesterase ($\mu\text{kat}/l$)	10.8	41.6	10.6	42.2	12.3	41.7	10.3	34.0	10.2	18.1*
Urine volume (ml)	3	2.4	3.5	1.9	3.7	2.2	4.6	2.5	7.2	5.2
Specific gravity (g/l)										
≤ 1040	1	0	1	0	0	0	1	0	5*	4*
≥ 1041	4	5	4	5	5	5	4	5	0	1

From Mellert et al. (1999d)

ALT, Alanine aminotransferase; F, female; M, male; MCHC, Mean corpuscular haemoglobin concentration; MCV, Mean corpuscular volume

* $p \leq 0.05$; ** $p \leq 0.01$

bilirubin in males, decreased glucose concentration in both sexes, and decreased globulin concentration in both sexes). The incidence of mucosal hyperplasia in the duodenum was increased at 500 and 1500 mg/kg. Reduced body-weight gains and fat storage in the liver were likely to be secondary to reduced food consumption, which may be related to poor palatability of the treated feed. Consequently, undue weight was not attached to these findings. The NOAEL was 100 mg/kg (equal to 9 mg/kg bw per day) on the basis of anaemia and associated findings, and mucosal hyperplasia at 500 mg/kg (equal to 42 mg/kg bw per day) (Mellert et al., 1999d).

Groups of 10 male and 10 female Wistar rats (aged 9 weeks) received pyraclostrobin (purity, 99%; in 0.5% carboxymethylcellulose) at a dose of 0, 40, 100 or 250 mg/kg bw per day, 5 days per week, for 4 weeks, in an application to the clipped dorsal surface. The test site, approximately 10% of the body surface area, was covered with a semi-occlusive dressing for 6 h after application. Rats were checked at least once daily for mortality and signs of toxicity, detailed clinical examinations were conducted at least once weekly, body weight, food consumption and food conversion efficiency were recorded once per week, and ophthalmoscopy was performed on animals in the control group and in the group at 250 mg/kg bw per day before sacrifice. At study termination, haematology, urine analysis and clinical chemistry parameters were assessed, animals were examined for gross pathology and organs (including uterus) were weighed. In the control group and in the group at 250 mg/kg bw per day only, a standard range of tissues (including nasal cavity, larynx, pharynx and treated skin) were examined microscopically. The uterus, vagina and treated skin were examined microscopically in all animals at 40 and 100 mg/kg bw per day and gross lesions were assessed in all animals affected.

Table 11. Organ weights and histology findings in rats receiving diets containing pyraclostrobin for 4 weeks

	Dietary concentration (mg/kg of feed)									
	0 (control)		20		100		500		1500	
	M	F	M	F	M	F	M	F	M	F
<i>Organ weights</i>										
Body weight (g)	297	183	302	184	301	186	281	176	241**	161*
Adrenal glands (mg)	80.4	84.4	80.4	91.4	79.6	86.4	78.6	85.2	73.6	72.6
Brain (g)	1.82	1.77	1.87	1.73	1.88	1.80	1.86	1.70	1.77	1.71
Kidneys (g)	2.47	1.73	2.53	1.70	2.37	1.71	2.36	1.64	2.06*	1.50
Thymus gland (mg)	424	307	444	297	427	273	357	270	330**	244
Relative weight ^a of brain	0.61	0.97	0.62	0.94	0.63	0.97	0.66	0.97	0.74**	1.06*
Relative weight ^a of liver	3.51	3.45	3.28	3.40	3.32	3.34	3.48	3.43	4.05*	4.36**
Relative weight ^a of spleen	0.21	0.25	0.25	0.24	0.24	0.28	0.28	0.33*	0.35*	0.38**
<i>Pathology</i>										
No. of animals examined	5	5	5	5	5	5	5	5	5	5
Duodenum										
Mucosal hyperplasia	0	1	0	0	0	0	4	2	4	4
Liver										
Fatty change	5	4	5	5	5	5	0	0	0	0
Hepatocellular hypertrophy	0	0	0	0	0	0	0	0	4	1
Spleen										
Extramedullary haematopoiesis	0	1	0	1	0	0	4	5	5	4

From Mellert et al. (1999d)

^aRelative organ weight = organ weight (g)/body weight (g) × 100

* $p \geq 0.05$; ** $p \geq 0.01$

M, Male; F, Female

There were no mortalities, no treatment-related effects on ophthalmology, haematology, clinical chemistry and urine analysis parameters and no abnormal findings in the open field observations for any animal. On the treated skin, clinical signs included scale formation in most females and some males at 250 mg/kg bw per day, and in some females at 100 mg/kg bw per day. Slight erythema was also seen in most females at 250 mg/kg bw per day. Body-weight gain was reduced by 8% in males at 250 mg/kg bw per day and food conversion efficiency was lower in males at 100 and 250 mg/kg bw per day. Absolute and relative uterus weights were increased by 27% and 30%, respectively, at 250 mg/kg bw per day. Epidermal thickening seen at 100 and 250 mg/kg bw per day was characterized by cloudy swelling of epidermal cells, associated with hyperkeratosis. In the uteri of rats at 250 mg/kg bw per day, dilation of the lumen was more common (two, one, two and six rats at 0, 40, 100 and 250 mg/kg bw per day, respectively; $n = 10$). After dermal administration of pyraclostrobin to rats for 4 weeks, the NOAEL for systemic toxicity for both sexes was 100 mg/kg bw per day on the basis of effects on the uteri of females and reduced body-weight gains in males at 250 mg/kg bw per day. Signs of dermal irritation occurred in all treated animals (Mellert et al., 1999c).

Groups of 10 male and 10 female Wistar rats (aged 42 days) were given diets containing pyraclostrobin (purity, 98.5%) at a concentration of 0, 50, 150, 500, 1000, or 1500 mg/kg of feed (equal to 0, 3.5, 11, 35, 69 and 106 mg/kg bw per day) for 3 months. Rats were checked at least once daily for mortality and signs of toxicity, and a comprehensive clinical examination was performed once weekly. Body weight and food consumption were recorded once weekly and water consumption was assessed daily. Haematology (including reticulocytes), urine analysis and clinical chemistry (excluding brain cholinesterase) parameters were assessed in all animals. At sacrifice, all animals were examined grossly and organ weights (excluding epididymides, heart and thymus gland) were recorded. A full range

of tissues was examined microscopically in the control group and at 1500 mg/kg. The lungs, liver, spleen, kidneys, stomach, duodenum, jejunum, ileum, sternum with marrow and bone marrow from the femur were examined microscopically in animals at 50, 150, 500 and 1000 mg/kg, and gross lesions were assessed in all animals affected per group.

There were no deaths or treatment-related clinical signs. At ≥ 500 mg/kg in males and at ≥ 1000 mg/kg in females, body-weight gain was reduced. Food consumption was consistently reduced in males, and intermittently in females at ≥ 500 mg/kg. Food conversion efficiency was also intermittently reduced at 1000 and 1500 mg/kg. Increased erythrocyte turnover was reflected in a marked increase in reticulocyte counts (41–94%) in males at ≥ 1000 mg/kg and in females at 1500 mg/kg, extramedullary haematopoiesis in both sexes at ≥ 150 mg/kg and in a slight anaemia in females at 1000 and 1500 mg/kg characterized by reductions of generally 10% or less in erythrocyte counts, haemoglobin concentrations and, at 1500 mg/kg, erythrocyte volume fraction. Other haematological effects consisted of increases in the values for MCV in both sexes at 1000 and 1500 mg/kg and females at 500 mg/kg (3–6%), prothrombin time in males at 1000 and 1500 mg/kg (11 and 13%), leukocyte counts in both sexes at 1500 mg/kg and females at 1000 mg/kg (14–71%), polymorphonuclear neutrophils in both sexes at 1000 and 1500 mg/kg (56–120%), and lymphocytes in females at 1000 and 1500 mg/kg (70–76%). Clinical chemistry changes consisted of increases in bilirubin in both sexes at 1500 mg/kg and males at 1000 mg/kg (30–95%), albumin in males at ≥ 500 mg/kg (5–6%) and erythrocyte cholinesterase activity in both sexes at 1500 mg/kg (23–33%), and in decreases in alanine aminotransferase activity in both sexes at ≥ 500 mg/kg (20–35%), alkaline phosphatase activity in both sexes at 1000 and 1500 mg/kg and females at 500 mg/kg (14–23%), globulin concentration in both sexes at 1000 and 1500 mg/kg (8–13%), concentration of triglycerides in males at 1000 and 1500 mg/kg (50–61%), cholesterol concentration in males at ≥ 500 mg/kg (19–29%), creatinine concentration in females at 1000 and 1500 mg/kg (10%) and serum cholinesterase activity in females at 1000 and 1500 mg/kg (41–49%). As alanine aminotransferase and alkaline phosphatase activities were also reduced in the 28-day and long-term studies in rats, the effect is clearly treatment-related but, given the small magnitude of the effect and as a slight reduction in these parameters is not normally associated with adverse organ or systemic effects, is unlikely to be toxicologically relevant. This conclusion is further supported by studies indicating that alanine aminotransferase (and alkaline phosphatase) activities can be affected by dietary status, as discussed later in this monograph. Males at 1000 and 1500 mg/kg had dark yellow to light red cloudy urine and females at 1500 mg/kg had cloudy urine. In females at 1000 and 1500 mg/kg, urine volume was increased by 48% and 130%, respectively, and specific gravity was decreased slightly. The number of crystals in the urinary sediment was slightly increased in males at 1500 mg/kg. Increased relative liver weights in both sexes at 1500 mg/kg and females at 1000 mg/kg (10–34%), kidney weights in both sexes at 1000 and 1500 mg/kg (8–15%), and spleen weights in both sexes at 1000 and 1500 mg/kg and females at 500 mg/kg (22–74%) were likely to be treatment-related as they correlated with histological and/or clinical pathology findings and/or were inconsistent with normal patterns of organ weight changes associated with reduced body-weight gain. Other organ weight differences were likely to be caused by reductions in body-weight gains (25–42%) in these groups. Thickening of the duodenal wall and discoloration of the spleen were more common at 1500 mg/kg. Microscopic examination demonstrated an increase in the incidence and/or severity of mucosal hyperplasia in the duodenum in both sexes at 1500 mg/kg and in males at 500 and 1000 mg/kg, hepatocellular hypertrophy in the liver of both sexes at 1500 mg/kg and in males at 500 and 1000 mg/kg and, in the spleen, distension of the sinusoids in both sexes at 1000 and 1500 mg/kg, extramedullary

Table 12. Haematology and clinical chemistry findings in rats given diets containing pyraclostrobin for 3 months

Parameter	Dietary concentration (mg kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
<i>Haematology</i>												
Erythrocytes (10 ¹² /l)	8.5	8.0	8.5	7.9	8.8	8.0	8.6	7.7	8.4	7.4***	8.2	7.1***
Haemoglobin (mmol/l)	9.7	9.2	9.5	9.3	9.8	9.3	9.7	9.3	9.5	8.7***	9.4	8.6***
Erythrocyte volume fraction (l/l)	0.43	0.41	0.43	0.41	0.44	0.42	0.44	0.42	0.44	0.40	0.43	0.39*
MCV (10 ⁻¹⁵ l)	50.6	51.5	50.1	52.0	50.2	52.1	51.3	53.9***	52.3*	53.8***	52.9**	54.8***
MCH (10 ⁻¹⁵ mol/l)	1.14	1.16	1.12	1.18	1.12	1.17	1.13	1.20***	1.14	1.19**	1.15	1.21***
MCHC (mmol/l)	22.5	22.6	22.3	22.6	22.3	22.4	21.9**	22.4	21.8***	22.2***	21.7***	22.2**
Reticulocytes (10 ⁻³ erythrocytes)	17	14	17	17	16	14	19	13	24**	15	33***	23***
Prothrombin time (s)	26.0	25.6	26.5	24.7	26.4	25.5	27.1	26.1	28.9***	27.5***	29.4***	26.3
Leukocytes (×10 ⁹ /l)	8.4	3.9	9.0	4.2	8.1	4.9	8.9	4.7	8.9	6.7***	9.6	6.6**
Neutrophils (×10 ⁹ /l)	0.66	0.68	0.71	0.81	0.60	0.68	0.78	0.71	1.25	1.06	1.45	1.25
Lymphocytes (×10 ⁹ /l)	6.96	2.84	7.53	2.97	6.74	3.74	7.50	3.58	7.03	5.00	7.37	4.83
<i>Clinical chemistry</i>												
ALT (μkat/l)	1.05	0.98	1.03	0.77**	0.91	0.89	0.73***	0.78*	0.68***	0.65***	0.79**	0.71**
AP (μkat/l)	5.6	4.5	5.7	4.1	5.9	4.4	5.3	3.8**	4.3**	3.8	4.3***	3.6***
Bilirubin (μmol/l)	1.7	2.2	1.7	1.9	1.8	1.9	2.2	1.9	2.7***	2.7	3.3***	2.9**
Albumin (g/l)	35.5	38.3	35.8	39.8	36.7	38.2	37.3**	38.6	37.8**	36.6	37.8***	37.2
Globulin (g/l)	31.6	30.6	33.1	32.5	32.6	30.6	31.2	29.4	29.2**	26.7**	27.5***	26.8**
Glucose (mmol/l)	7.9	7.9	8.1	8.1	7.6	7.7	7.6	7.2	7.3*	7.0**	7.2**	7.3
ECHE (μkat/l)	18.4	16.7	20.6*	20.9	18.7	20.0	20.8	18.4	20.2	21.1	22.6**	22.2
SCHE (μkat/l)	10.0	47.9	9.8	57.5	10.8	49.9	10.6	41.2	10.4	28.5***	10.3	24.3***
Triglyceride (mmol/l)	3.8	1.6	3.6	2.6	4.4	2.3	2.9	2.2	1.9**	1.5	1.5***	2.1
Creatinine (μmol/l)	49.8	55.6	49.5	57.3	50.3	56.1	48.9	52.8	48.9	50.0**	48.4	50.3**
Cholesterol (mmol/l)	2.3	1.8	2.2	2.1	1.9	1.8	1.8*	1.8	1.7***	1.7	1.6***	1.8

From Mellert et al. (1999a, 1999b)
ALT, Alanine aminotransferase; AP, Alkaline phosphatase; ECHE, erythrocyte cholinesterase; F, Female; M, Male; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; MCV, Mean corpuscular volume; SCHE, serum cholinesterase
* $p \leq 0.05$; ** $p \leq 0.02$; *** $p \leq 0.002$

haematopoiesis in both sexes at ≥ 150 mg/kg and histiocytosis in both sexes at ≥ 150 mg/kg. Fat storage in the liver was diminished in both sexes at ≥ 150 mg/kg, which is likely to reflect the reduced weight gains observed at ≥ 500 mg/kg. In two long-term studies in rats, at intakes of pyraclostrobin of up to 200 mg/kg (9 mg/kgbw per day), no effects on splenic extramedullary haematopoiesis or histiocytosis, or on fat storage in the liver were seen. Consequently the low incidences of these findings at 150 mg/kg were considered to be incidental to treatment. The NOAEL was 150 mg/kg (10.7 mg/kgbw per day in males and 12.3 mg/kgbw per day in females) on the basis of reductions in body weight and food consumption, effects on clinical chemistry parameters, liver hypertrophy, and mucosal hypertrophy in the duodenum at ≥ 500 mg/kg (Mellert et al., 1999a, 1999b).

Rabbits

Groups of five male and five female (not pregnant) Himalayan rabbits (Chbb:HM from Charles River Laboratories Germany) were given pyraclostrobin (in a 0.5% aqueous solution of carboxymethylcellulose) at a dose of 0 or 4 mg/kgbw per day for 1 week by gavage. Pyraclostrobin was also administered in the diet at concentrations calculated to provide doses of 0 or 4 mg/kgbw per day for 1 week. Animals were quarantined and acclimatized

Table 13. Organ weights in rats receiving diets containing pyraclostrobin for 3 months

Organ	Dietary concentration (mg/kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
<i>Absolute weights</i>												
Body (g)	439	230	444	243	424	211	406*	224	370**	212	319**	210**
Adrenals (mg)	86.3	99.9	81.5	90.9	84.5	94.5	71.8**	88.4*	72.6**	85.2**	71.3**	76.6**
Brain (g)	2.06	1.82	2.03	1.86	2.07	1.88*	2.02	1.82	2.02	1.77*	1.99	1.78*
Kidneys (g)	2.98	1.79	3.05	1.86	2.87	1.88	2.86	1.84	2.76	1.84	2.48**	1.76
Liver (g)	15.0	6.8	14.0	7.3	13.2*	7.1	13.1*	6.9	12.3**	7.3	11.9**	8.3**
Ovaries (mg)	—	96.8	—	107.1	—	101.9	—	103.6	—	102.3	—	112.9
Spleen (g)	0.87	0.56	0.90	0.63	0.84	0.60	0.85	0.65*	0.95	0.74**	1.02*	0.88**
Testes (g)	3.58	—	3.57	—	3.57	—	3.52	—	3.74	—	3.68	—
<i>Relative (to body) weights*</i>												
Adrenals	0.020	0.044	0.018	0.038*	0.020	0.040	0.018	0.039	0.020	0.039	0.022*	0.037**
Brain	0.47	0.80	0.46	0.77	0.49	0.81	0.50	0.81	0.55**	0.82	0.62**	0.85
Kidneys	0.68	0.78	0.68	0.77	0.68	0.80	0.71	0.82*	0.75*	0.85*	0.78**	0.84*
Liver	3.4	2.9	3.1	3.0	3.1	3.0	3.2	3.1*	3.3	3.3**	3.7	3.9**
Ovaries	—	0.042	—	0.044	—	0.044	—	0.046	—	0.047	—	0.054**
Spleen	0.20	0.24	0.20	0.26	0.20	0.26	0.21	0.29*	0.26**	0.34**	0.32**	0.42**
Testes	0.82	—	0.81	—	0.85	—	0.87	—	1.01**	—	1.15**	—

From Mellert et al. (1999a, 1999b)
F, Female; M, Male
*Relative organ weight = organ weight (g)/body weight (g) × 100; * *p* < 0.05; ** *p* < 0.01

Table 14. Pathology findings in rats receiving diets containing pyraclostrobin for 3 months

Finding	Dietary concentration (mg/kg of feed)											
	0 (control)		50		150		500		1000		1500	
	M	F	M	F	M	F	M	F	M	F	M	F
No. of animals examined	10	10	10	10	10	10	10	10	10	10	10	10
<i>Gross pathology</i>												
Duodenum, thickening of wall	0	0	0	0	0	0	0	0	0	2	10	10
Spleen, discoloration	0	0	0	0	0	0	0	0	1	1	6	5
<i>Histopathology</i>												
Duodenum, mucosal hyperplasia												
Grade 1 ^a	2	2	1	1	1	1	3	1	4	0	1	6
Grade 2 ^a	0	0	0	0	0	1	1	0	1	1	9	4
Total	2	2	1	1	1	2	4	1	5	1	10	10
Liver												
Diffuse fatty change	10	4	8	7	9	5	6	2	2	1	0	0
Hepatocellular hypertrophy	0	0	0	0	0	0	3	0	6	0	10	4
Spleen												
Distension of sinusoids	0	0	0	0	0	0	1	2	10	8	8	10
Extramedullary haematopoiesis	2	0	0	0	3	3	1	3	2	9	3	9
Histiocytosis	0	0	0	0	1	1	3	2	6	7	10	7

From Mellert et al. (1999a, 1999b)
F, Female; M, Male
^aGrade 1 = minimal in severity/very few in number/very small in size, grade 2 = slight in severity/few in number/small in size, grade 3 = moderate in severity and size/moderate to several in number.

to standard laboratory conditions for approximately 1 week before the start of the study. Food and water were available ad libitum. Food consumption was determined daily and body weight was recorded on days 0, 2, 4, 6 and 7. Animals were examined for mortality and clinical signs of toxicity at least once per day. At the end of the experiment, animals were sacrificed for necropsy. There were no clinical signs of toxicity and no abnormal

macroscopic changes observed at necropsy. From the first day of dosing, mean food consumption was about 15–40% lower in both groups of treated males, but the differences from values in the relevant control groups were not statistically significant. Similarly, in both groups of females mean food consumption was 20–40% lower from the beginning of dosing until day 5. Thereafter, it was similar in control and treated groups. Although there were some apparent differences between mean food consumption and body-weight gains in control and treated groups, there was considerable variation between individuals in all groups and also variation in values obtained for any one individual on different days. Table 15 provides the ranges of values for food consumption and body-weight gain measured in control and treated animals. There was no clear effect of treatment with pyraclostrobin and it is probable that the apparent variation observed was a part of the normal biological variation for this species. This was a supplementary study initiated to address the appropriateness of using an apparently lower body-weight gain in treated animals in a study of developmental toxicity in rabbits as an appropriate end-point on which to base an acute RfD. In conjunction with similar studies in mice and rats, this study was intended to demonstrate the species-specific variability in food intake and body-weight gains of rabbits. Because of the limited parameters examined in this study, it was not adequate for the purposes of risk assessment and a NOAEL could not be identified (Schneider & Hellwig, 2002).

Dogs

Groups of five male and five female beagle dogs (aged 7–8 months) were given diets containing pyraclostrobin (purity, 97.1%) at a concentration of 0, 100, 200, or 450 mg/kg of feed (equal to 0, 2.8, 5.8 and 13 mg/kg bw per day) for 3 months. Dogs were checked at least once daily for mortality, and clinical signs of toxicity were assessed at least once each working day. Body weight was recorded weekly and food consumption was recorded daily. Haematology (including activated partial thromboplastin time) and clinical chemistry parameters (excluding brain and erythrocyte cholinesterase activity) were assessed in all animals on days 41/43 and 90, and an ophthalmological examination was performed at the end of the study. Urine analysis parameters were assessed in all animals on days 37/38 and 86/87.

Table 15. Variations in food consumption and body-weight changes in rabbits

	Dose			
	Gavage control	4 mg/kg bw per day, by gavage	Diet control	4 mg/kg bw per day via the diet
<i>Food consumption</i>				
Minimum, g/animal/day (M/F)	14/36	26/26	12/12	21/21
Maximum, g/animal/day (M/F)	134/144	126/108	112/112	82/93
<i>Body-weight changes</i>				
Days 0–7 (M/F)	57/41	–10/27	–8/–21	–48/–26
Extreme values:				
Males				
Days 0–2 (max/min)	51/–18	22/–6	180/–165	1/–38
Days 2–4 (max/min)	11/–53	13/–26	2/–137	17/–46
Days 4–6 (max/min)	95/–46	4/–90	79/–17	34/–46
Days 6–7 (max/min)	18/1	60/–24	43/15	4/–21
Females				
Days 0–2 (max/min)	55/6	75/–27	56/–76	21/–55
Days 2–4 (max/min)	40/1	35/–26	21/–30	10/–65
Days 4–6 (max/min)	30/–30	20/–24	24/–64	21/–27
Days 6–7 (max/min)	20/–30	29/–15	55/–10	88/–6

From Schneider & Hellwig (2002)
F, Female; M, Male; max/min, maximum/minimum

At sacrifice, all dogs were examined grossly, their tissues (including gall bladder, but excluding seminal vesicles) were examined microscopically and gross lesions were assessed. Organ weights (including parathyroid and thyroid glands, but excluding heart, spleen and thymus gland) were recorded.

There were no deaths, but vomiting (10 out of 10 dogs at 450 mg/kg until week 2) and diarrhoea (10 out of 10 dogs at 450 mg/kg during most of the study) were observed. At 200 mg/kg, diarrhoea also occurred in two out of five males during week 5 only, and sporadically throughout the study in four out of five females. As diarrhoea was observed in a 12-month study in dogs at 400 but not at 200 mg/kg, the occurrences of diarrhoea at 200 mg/kg in this study were regarded as being of no toxicological concern, owing to their isolated occurrence (week 5 only) in almost all affected dogs, or the irregular occurrence in a single animal. All other observations, discussed in the following paragraphs, relate to the treatment at 450 mg/kg only, unless specifically indicated otherwise. Females lost 2% (200 g) of their body weight, and males at 200 and 450 mg/kg gained substantially less weight (up to 31%) than did the controls. The effects in males were greatly influenced by one animal at 200 mg/kg that gained no weight and one at 450 mg/kg that lost weight (1%) during the study. Consequently, the finding in males is of questionable toxicological significance, particularly as no effect on body-weight gains was observed at 200 mg/kg in a subsequent 12-month study in dogs. In females, food consumption (9%) and food conversion efficiency were reduced. There were no treatment-related effects on ophthalmology.

Platelet counts were increased by 47–60% in females, and concentrations of total protein, albumin, globulin (both sexes, 5–12%) and glucose (females, 9–13%) were reduced, and absolute and relative epididymides weights were slightly increased (10–14%). A reduction in absolute and relative liver weights in females is consistent with, and likely to be secondary to, reduced food intake and weight gain. Urine analysis parameters were unaffected by treatment.

In the duodenum of both sexes, thickening of the wall and mucosal hypertrophy, which was characterized by an increased ratio of cytoplasm to nuclei in the villi and a hyperplastic aspect in epithelial cells, were observed. The NOAEL was 200 mg/kg (5.8 mg/kg bw per day) on the basis of increased platelet counts in females, decreased concentrations of total protein, albumin, and globulin in both sexes, decreased concentration of glucose and liver weights in females, and thickening of the wall and mucosal hypertrophy in the duodenum of both sexes at 450 mg/kg (Menges et al., 1999).

Groups of five male and female beagle dogs (aged 5–8 months) were given diets containing pyraclostrobin (purity, 98.7%) at a concentration of 0, 100, 200, or 400 mg/kg of feed (equal to 0, 2.7, 5.4 and 11 mg/kg bw per day) for 12 months. Animals were individually housed in kennels in controlled conditions. Each day, 700 g of food ration was offered to each dog, and water was provided *ad libitum*. Dogs were checked at least once daily for mortality, and clinical signs of toxicity were assessed at least once each weekday. Body weight and food conversion efficiency were recorded/calculated weekly, while food consumption was recorded daily. An ophthalmological examination was performed at the end of the study. Haematology (including activated partial thromboplastin time) and clinical chemistry (excluding brain, erythrocyte and serum cholinesterase activities) and urine analysis parameters were assessed in all animals at 3, 6 and 12 months. After 12 months of treatment, all dogs were sacrificed and examined grossly. Tissues (including gall bladder, but excluding femur with joint/glenoid surface and seminal vesicles) were examined micro-

Table 16. Observations in dogs given diets containing pyraclostrobin for 3 months

Parameter	Dietary concentration (mg/kg of feed)							
	0 (control)		100		200		450	
	M	F	M	F	M	F	M	F
<i>Haematology</i>								
Platelets (×10 ⁹ /l)								
Day 41/43	290	264	265	313	294	293	310	389
Day 90	272	258	265	290	296	300	303	412**
<i>Clinical chemistry</i>								
Total protein (g/l)								
Day 41/43	57.9	56.4	56.3	56.0	56.2	55.5	53.2	52.0
Day 90	59.3	58.2	58.0	57.8	57.6	58.1	53.5	53.2**
Albumin (g/l)								
Day 41/43	34.0	33.1	33.2	34.3	33.4	33.5	31.6	31.4
Day 90	31.6	30.9	30.9	31.1	30.6	30.8	29.1	28.7
Globulin (g/l)								
Day 41/43	23.9	23.2	23.1	21.7	22.8	22.0	21.6	20.5
Day 90	27.6	27.3	27.1	26.7	26.9	27.3	24.4	24.5
Glucose (mmol/l)								
Day 41/43	6.3	6.4	6.3	6.3	6.4	6.1**	5.9	5.8**
Day 90	6.0	6.2	6.0	6.0	6.0	5.8*	5.8	5.3**
<i>Absolute organ weights</i>								
Body (g)	12 980	12 540	13 900	12 860	12 580	12 300	12 640	10 480
Epididymides (g)	3.79	—	3.54	—	3.44	—	4.17	—
Liver (g)	388	361	418	382	386	320	361	286
<i>Relative (to body) organ weights^a</i>								
Epididymides	0.029	—	0.025	—	0.027	—	0.033	—
Liver	2.99	2.83	3.01	2.98	3.08	2.60	2.86	2.72
<i>Pathology</i>								
No. of animals examined	5	5	5	5	5	5	5	5
<i>Gross findings</i>								
Duodenum, thickening of wall	0	0	0	0	0	0	2	2
<i>Microscopic findings</i>								
Duodenum, mucosal hypertrophy	0	0	0	0	0	0	2	1

From Menges et al. (1999)
^aRelative organ weight = organ weight (g)/body weight (g) × 100
* *p* < 0.05, ** *p* < 0.01
M, Male; F, Female

scopically and gross lesions were assessed in all animals affected per group. Organ weights (including parathyroid and thyroid glands, but excluding heart, spleen and thymus gland) were recorded.

There were no effects on the incidence of premature mortality, ophthalmology or pathology findings, or on urine analysis parameters, and treatment-related effects were only seen at 400 mg/kg. In all or most animals, vomiting was seen in week 1, and diarrhoea was seen throughout the study. Slight body-weight losses of 1–2% occurred in both sexes until day 7. Thereafter, body-weight gain in males was greater by 53% than that in controls, while in females, body-weight gain was reduced by 48% throughout the entire study. In females, food consumption was reduced by 11%. Leukocyte counts (males) and platelet counts (both sexes) were increased (23–53%). Isolated reductions in erythrocyte volume fraction and haemoglobin concentration were recorded in males at day 180 and females at day 90, but these were concluded to be incidental to treatment owing to the absence of similar findings

at other observation times. Concentrations of total protein, albumin, globulin and cholesterol were reduced (5–35%) in both sexes.

Absolute liver weight was reduced by 18% in females, but relative liver (to body) weights were comparable to those of the controls, and no histological alterations were observed. It was therefore concluded that the lower liver weight was secondary to the reduced weight gains in these animals, rather than to a direct toxicological effect on the liver. Although absolute kidney weights were increased by 16–21% in males at all doses, the range of kidney weights for treated animals (54–76 g) was within the range for historical controls from 21 feeding studies (52–76 g); for the control animals, the range was 46–58 g with the weight in one animal being only 46 g, which is below the range for historical controls. On this basis, and in the absence of a dose–response relationship, effects on biochemical markers (creatinine and urea) and pathology findings, it was concluded that the apparent increases in kidney weights were incidental to treatment. The NOAEL was 200 mg/kg, equal to 5.4 mg/kg bw per day, on the basis of clinical signs of toxicity, reduced body-weight gains, and haematological and clinical biochemistry effects at 400 mg/kg (Schilling et al., 1999a).

Table 17. Principle observations in a 12-month study in dogs given diets containing pyraclostrobin

Parameter	Dietary concentration (mg/kg of feed)							
	0 (control)		100		200		400	
	Males	Females	Males	Females	Males	Females	Males	Females
<i>Haematology and clinical chemistry</i>								
White blood cells ($\times 10^9/l$)								
Day 89/90	9.9	12.5	11.5	11.8	11.2	12.8	12.2	10.7
Day 180/181	9.0	10.7	10.3	10.9	9.6	10.3	12.8**	11.7
Day 362/363	8.3	10.7	9.3*	11.5	8.7	11.7	12.7**	11.4
Platelets ($\times 10^9/l$)								
Day 89/90	268	295	284	272	253	331	366**	351
Day 180/181	283	297	290	306	266	335	348**	386
Day 362/363	304	319	274	302	267	349	392**	401
Total protein (g/l)								
Day 89/90	58.3	58.0	59.0	58.6	56.9	57.1	52.6**	51.4**
Day 180/181	57.3	57.4	58.4	59.5	58.3	56.4	53.1**	51.5**
Day 362/363	61.8	62.0	61.6	63.3	62.0	59.4	53.5**	55.4**
Albumin (g/l)								
Day 89/90	34.9	35.6	35.0	36.4	34.4	34.9	31.7**	32.0
Day 180/181	30.2	30.5	30.3	31.0	30.6	30.2	27.9**	27.7
Day 362/363	31.0	30.9	30.2	32.4	31.1	30.6	27.0**	29.5
Globulin (g/l)								
Day 89/90	23.4	22.5	24.1	22.1	22.4	22.2	20.9	19.4
Day 180/181	27.1	26.9	28.1	28.5	27.7	26.2	25.2	23.8**
Day 362/363	30.8	31.1	31.5	30.9	30.9	28.8	26.6	25.9**
Cholesterol (mmol/l)								
Day 89/90	4.8	4.5	5.2	4.6	4.5	4.3	3.3**	3.4
Day 180/181	4.3	4.8	4.8	5.5	4.4	4.1	3.2**	3.1**
Day 362/363	4.6	4.6	4.6	5.4	4.4	4.4	3.2**	3.2**
<i>Organ weights</i>								
Body (g)	12 960	12 680	12 661	13 480	13 360	13 060	13 101	11 140
Kidney (g)	54.1	51.4	62.9*	53.9	65.0*	51.6	65.4*	52.7
Liver (g)	383	344	388	387	370	367	349	282
Relative ^a kidney weights	0.42	0.41	0.50	0.40	0.49	0.40	0.50	0.48
Relative ^a liver weights	2.99	2.73	3.06	2.87	2.76	2.82	2.66	2.55

From Schilling et al. (1999a)

^aRelative organ weight = organ weight (g)/body weight (g) \times 100

* $p \leq 0.05$; ** $p \leq 0.02$

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 50 male and 50 female B6C3F₁ mice (aged 47–51 days) were given diets containing pyraclostrobin (purity, 97.1%) at a concentration of 0, 10, 30, or 120 mg/kg of feed (equal to 0, 1.4, 4.1 and 17 mg/kg bw per day), and to females only at 180 mg/kg (equal to 33 mg/kg bw per day), for 18 months. Animals were individually housed in cages in controlled conditions and food and water were available ad libitum. Mice were checked at least once daily for mortality and signs of toxicity and a comprehensive clinical examination was performed once per week. Body weight, food consumption and food conversion efficiency were recorded/calculated weekly for the first 13 weeks and at 4-week intervals thereafter. Water consumption was checked daily. Blood was collected from non-fasted animals at 12 months and fasted animals at 18 months, blood smears were prepared and differential blood counts were assessed in animals in the control group, at 120 mg/kg (males only) and at 180 mg/kg (females only). At sacrifice, all animals were examined grossly, organ weights (excluding epididymides, heart, spleen and thymus gland) were recorded, and histology was performed on a comprehensive range of tissues (including gall bladder) from animals in the control group, at 120 mg/kg (males) and at 180 mg/kg (females). The thymus gland, lungs, liver, kidneys, stomach and duodenum were examined in animals at 10 and 30 mg/kg and in females at 120 mg/kg, and gross lesions were assessed in all animals affected. Mortality, clinical signs, food conversion efficiency and water consumption were unaffected by treatment. Body-weight gain was reduced (17–28%) in all treated groups over the study, but in both sexes at 10 and 30 mg/kg and in females at 120 mg/kg, the reductions in body-weight gain were largely caused by body-weight losses that occurred from day 399/427, which were associated with reduced food consumption (11–21%) in all treated animals from day 427. However, reductions in body-weight gain in males at 120 mg/kg and females at 180 mg/kg occurred from the end of the first week of treatment and were generally statistically significantly different from those in the control group throughout the study, and as such, only these effects were considered to be of toxicological significance.

Circulating monoblasts were observed in 15% of females at 180 mg/kg, but in no control animals. Differential blood counts were not assessed in females from the other treated groups. Absolute and relative liver weights were reduced in treated male groups, reflecting reduced weight gains and feed intake. Absolute liver weights were largely unaffected in females, but the relative weight was increased at 180 mg/kg; this is likely to reflect a treatment-related effect.

There were no treatment-related pathology or neoplastic findings. Although the limited range of investigations carried out at doses other than the control and highest dose for each sex would normally preclude identification of a NOAEL, given the absence of gross or histopathological findings at the highest dose, the NOAEL in this 18-month carcinogenicity study in mice was 30 mg/kg (4.1 mg/kg bw per day for males) on the basis of reduction of body weights in males at 120 mg/kg during the first year of the study. During the last 6 months of the study, food consumption, body weight and body-weight change were significantly reduced at all doses. However, these effects at the end of the treatment period, after the growth phase of the animals, were without any dose–response relationship and were not regarded as treatment-related at doses <120 mg/kg in males and 180 mg/kg in females. (Mellert et al., 1999g).

Table 18. Principal findings in an 18-month study in mice given diets containing pyraclostrobin

Organ weights	Dietary concentration (mg/kg of feed)								
	0 (control)		10		30		120		180
	M	F	M	F	M	F	M	F	F
Body (g)	39	35	37**	33*	37**	35	35**	32**	31**
Liver (g)	1.59	1.34	1.34**	1.25	1.41*	1.27	1.29**	1.23	1.28
Liver, relative ^a to body, weight	4.05	3.87	3.67**	3.86	3.85	3.72	3.68	3.92	4.12**

From Mellert et al. (1999g)

F, Female; M, Male

* $p < 0.05$, ** $p < 0.02$

^aRelative organ weight = organ weight (g)/body weight (g) \times 100

Rats

Groups of 20 male and female Wistar rats (aged 42 days) were given pyraclostrobin (purity, 97.1%) at a concentration of 0, 25, 75, or 200 mg/kg of feed (0, 1.1, 3.4 and 9 mg/kg bw per day for 24 months). Animals were individually housed in cages in controlled conditions and food and water were available ad libitum. Rats were checked at least once daily for mortality and signs of toxicity, and a comprehensive clinical examination was performed once weekly. Body weight, food consumption and food conversion efficiency were recorded or calculated weekly for the first 13 weeks and at 4-week intervals thereafter. An ophthalmological examination was performed at the end of the study. Haematology, clinical chemistry (excluding brain and erythrocyte cholinesterase activities), and urine analysis parameters were assessed at approximately 3, 6, 12, 18 and 24 months. After sacrifice, animals were examined for gross pathology, a full range of tissues was examined histologically, gross lesions were assessed in all animals affected per group and organ weights (excluding epididymides, heart, spleen and thymus gland) were recorded.

Treatment did not affect mortality, clinical signs, food consumption, food conversion efficiency, haematology or ophthalmology. Over approximately the first 18 months of the study, body-weight gain was reduced by 11–14% in males and females at 200 mg/kg. Alanine aminotransferase in males and alkaline phosphatase in both sexes were slightly but significantly reduced at 200 mg/kg. Although these effects were clearly treatment-related, and consistent with effects seen in the 28-day and 3-month studies in rats, given the direction and small magnitude of the changes it was concluded that they were of minimal toxicological significance. This conclusion was further supported by studies indicating that alanine aminotransferase (and alkaline phosphatase) activities can be affected by dietary status, as discussed later in this monograph. Transient variations in aspartate aminotransferase and serum cholinesterase activities in treated groups were not considered to be toxicologically significant. Concentrations of protein in the urine were slightly increased in treated rats, particularly males, but this was not associated with histopathological changes and has not been observed in other studies in rats. Consequently, the finding was discounted as being incidental to treatment. In males at 200 mg/kg, slight increases in absolute testes and adrenal gland weights were not considered to be toxicologically significant, because the effects were influenced by the organ weight values of only one to two animals. Tubular degeneration of the testes occurred in one out of 20, seven out of 20, seven out of 20 and six out of 20 males in the control group, at 25 mg/kg, 75 mg/kg and 200 mg/kg, respectively. Given the absence of a dose–response relationship or of similar effects in other studies in rats, including another 2-year study and a two-generation study of reproductive toxicity,

Table 19. Principal findings in a 2-year study in rats given diets containing pyraclostrobin

Clinical chemistry parameter	Dietary concentration (mg/kg of feed)							
	0 (control)		25		75		200	
	Males	Females	Males	Females	Males	Females	Males	Females
ALT (μkat/l)	1.08	1.12	1.05	1.00	1.07	1.00	0.93**	0.94
ALP (μkat/l)	4.72	3.72	5.62*	3.85	5.28*	3.62	4.28*	2.91**

From Mellert et al. (1999c)
ALT, Alanine aminotransferase; ALP, Alkaline phosphatase
* $p \leq 0.05$; ** $p \leq 0.02$; *** $p \leq 0.002$

reported below, it was concluded that this finding was incidental to treatment. There were no treatment-related neoplastic findings. The NOAEL was 75 mg/kg (3.4 mg/kg bw per day) on the basis of reduced body-weight gains at 200 mg/kg (Mellert et al., 1999e).

Groups of 50 male and female Wistar rats (aged 42 days) were given diets containing pyraclostrobin (purity, 97.1%) at a concentration of 0, 25, 75, or 200 mg/kg of feed (equal to 0, 1.2, 3.4 and 9 mg/kg bw per day) for 24 months. Animals were individually housed in cages in controlled conditions, and food and water were available ad libitum. Mortality and signs of toxicity were checked at least once daily, a comprehensive clinical examination was performed once weekly, and body weight, food consumption and food conversion efficiency were recorded/calculated weekly for the first 13 weeks and at 4-week intervals thereafter. Blood was collected from fasted animals at 24 months and differential blood counts were assessed in the control group and at 200 mg/kg. After 24 months of treatment, all rats were fasted, sacrificed and necropsied. All animals were examined for gross and histopathology, gross lesions were assessed in all animals affected per group and organ weights (excluding epididymides, heart, spleen and thymus gland) were recorded.

Treatment did not affect the nature or incidence of clinical signs or values for haematology parameters. Treatment-related effects were observed only at 200 mg/kg. Mortality was increased in males only, with 32 out of 50 deaths at 200 mg/kg compared with 22 out of 50 in controls. Body-weight gain was reduced by 10% in males over the first 18 months and by 22% in females over the entire study, but food consumption was only slightly reduced, by approximately 4%, in females up to day 91. In females, absolute liver weight was reduced (10%), but relative liver weight remained comparable to that of controls and no histological alterations were noted in female liver. It was therefore concluded that the decreased absolute liver weight was secondary to reduced weight gain, rather than a direct toxicological effect. The incidences of liver necrosis and liver adenomas were increased in males, but the incidence of liver carcinomas was unaffected. In the long-term study in rats, reported above, which was conducted concurrently with this study of carcinogenicity, the incidence of liver adenomas was lower in treated groups than in the controls (males: four, two, two, one; females: one, 0, 0, one; $n = 20$ for each group), liver carcinomas did not occur in a dose-related manner, as was also the case in the previous study (males: 0, two, two, 0; females: 0 for all groups; $n = 20$ for each group), and the incidence of tumours overall was similar in all groups. On this basis, undue weight was not attached to the apparent increase in liver adenomas in males at 200 mg/kg. Erosion and ulcers in the glandular stomach were increased in males. The NOAEL was 75 mg/kg (3.4 mg/kg bw per day) on the basis of reduced body-weight gain in both sexes, and altered liver and stomach histology in males at 200 mg/kg (Mellert et al., 1999f).

Table 20. Principal findings in a 2-year study of carcinogenicity in rats

Parameter/finding	Dietary concentration (mg/kg of feed)							
	0 (control)		25		75		200	
	M	F	M	F	M	F	M	F
<i>Organ weights</i>								
Body (g)	658	394	656	379	668	368	628	339**
Liver weight (g)	19.8	12.9	19.8	12.7	19.1	12.5	19.2	11.5**
Liver relative ^a (to body) weight	3.02	3.28	3.05	3.36	2.87	3.40	3.05	3.41
<i>Pathology</i>								
No. of animals examined	50	50	50	50	50	50	50	50
Liver								
Necrosis	1	3	2	4	2	2	10	3
Adenoma	4	3	7	3	5	0	11	5
Carcinoma	4	0	3	0	5	0	3	0
Stomach								
Erosion	2	3	5	3	7	4	10	3
Ulcers	2	1	2	2	2	1	7	1

From Mellert et al. (1999f)

F, Female; M, Male

^aRelative organ weight = organ weight (g)/body weight (g) × 100** $p \leq 0.01$ **Table 21. Results of studies of genotoxicity with pyraclostrobin**

End-point	Test object	Concentration or dose (solvent/vehicle)	Results	Reference
<i>In vitro</i>				
Reverse mutation	<i>S. typhimurium</i> <i>E. coli</i>	20–5000 µg/plate, ±S9 (in DMSO)	Negative	Engelhardt & Hoffman (1997)
Forward mutation	Chinese hamster ovary cells, <i>Hgpri</i> locus	0.625–20 µg/ml, ±S9	Negative	Engelhardt & Hoffman (1998a)
		3–8 µg/ml, ±S9	Negative	Englehardt (2000a)
		& 1.25–20 µg/ml, ±S9 (DMSO)	Negative	
Chromosomal aberration	Chinese hamster V79 cells	6.25–25 µg/ml, ±S9	Negative	Engelhardt & Hoffman (1999)
		0.005–0.100 µg/ml, –S9	Negative	
		3.125–12.5 µg/ml, +S9 (DMSO)	Negative	
Unscheduled DNA synthesis	Rat hepatocytes	0.01–0.5 µg/ml	Negative	Engelhardt & Hoffman (1998b),
		0.004–0.5 µg/ml (DMSO)	Negative	Englehardt (2000b)
<i>In vivo</i>				
Micronucleus formation	Mouse bone-marrow cells	75–300 mg/kg bw (olive oil)	Negative	Engelhardt & Hoffman (1998c)

S9, 9000 × g supernatant fraction of rodent liver

2.4 Genotoxicity

Pyraclostrobin (purity, 98.2%) was evaluated for potential genotoxicity in vitro in tests for mutagenicity in bacterial and mammalian cells, for chromosome damage (clastogenicity) and for unscheduled DNA synthesis. The results of these studies demonstrated the absence of a genotoxic effect. In vivo, the test substance was assessed for the induction of micronucleus formation in mice. The result of this study showed that pyraclostrobin does not exhibit a chromosome-damaging potential. It was therefore concluded that pyraclostrobin has no mutagenic or genotoxic properties either in vitro or in vivo.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of 25 male and female Wistar rats (aged 35 days) were given diets containing pyraclostrobin (purity, 98.7%) at a concentration of 0, 25, 75, or 300 mg/kg of feed (equal to 0, 2.7, 8.2 and 33 mg/kg bw per day) over two generations. Treatment was initiated at least 74 days before the mating of F_0 rats (1:1) and continued through two generations. Females were allowed to litter their pups, and litters were culled on postnatal day 4 to eight pups/litter (ideally, four males and four females), where possible. Pups were weaned on postnatal day 21, after which F_0 parental animals were sacrificed. Twenty-five F_1 rats of each sex per group were selected (each litter was taken into account) for mating of the adult F_1 generation, following the same protocol as described above for the F_0 mating. Reproductive performance was assessed using the following parameters: male mating index, male fertility index, female mating index, female fertility index, gestation index, live birth index, and postimplantation loss. All pups in the F_1 and F_2 litters were examined as soon as possible after birth to determine the number of live and stillborn pups in each litter. Pups were examined daily for clinical signs. The number of live pups/litter was calculated on postnatal days 4, 7, 14 and 21. The sex ratio was calculated on postnatal days 0 and 21. Pup body weights were recorded on postnatal days 1, 4, 7, 14 and 21. During the study, rats were individually housed in cages in controlled conditions and food and water were available ad libitum. In F_0 and F_1 parental animals, body weight and food consumption were recorded weekly and throughout gestation (days 0, 7, 14 and 20) and lactation (1, 4, 7, 14 and/or 21). Estrous cycle (length/normality) was assessed daily in F_0 and F_1 female parental rats for a minimum of 3 weeks before mating and was continued until females exhibited evidence of mating. At necropsy, a vaginal smear was taken to determine the stage of the estrous cycle for each F_0 and F_1 female. The weights of the right testis and cauda epididymis from F_0 and F_1 males of all groups were recorded and the following sperm parameters were determined: motility, morphology, and head count (cauda epididymis and testis).

All F_0 and F_1 parental animals were examined macroscopically. Organs (standard test parameters plus pituitary and prostate glands, seminal vesicles with coagulating glands and fluids and uterus with cervix uteri and oviducts, but excluding the heart) were weighed, and the kidneys from rats in all groups were examined microscopically. The vagina, cervix uteri, uterus, ovaries, oviducts, pituitary gland, left testis and epididymis, prostate gland, seminal vesicles, coagulating glands, liver, thymus gland and adrenal glands were assessed in all control and 300 mg/kg animals and in all animals at 25 and 75 mg/kg suspected of impaired fertility. A quantitative assessment of primordial, growing and antral follicles in the ovaries was performed for all F_0 and F_1 parental animals in the control group and at the highest dose. Gross lesions were assessed in all animals affected per group. F_1 and F_2 pups were sexed and weighed on the day after birth and on postnatal days 4, 7, 14 and 21. Pup survival was also recorded. At necropsy, all pups were examined macroscopically, and brain, spleen and thymus gland weights were recorded in one pup of each sex per litter. Sexual maturation (day of preputial separation/vaginal opening) was assessed in all pups selected for the F_1 parental generation.

In the F_0 generation, one female at 300 mg/kg died on the first day of lactation, but there were no mortalities in the F_1 generation. There were no treatment-related clinical signs in either generation. At 300 mg/kg, slight reductions in food consumption (up to 12% in

both generations during pre-mating and F₁ animals during gestation) and body-weight gain (up to 50% in F₀ animals during pre-mating and lactation) occurred in adult animals at times during the respective observation periods. In both generations, there were no effects on fertility, gestation, parturition or pup survival. Body-weight gain in F₁ and F₂ pups was reduced (12–15%) at 300 mg/kg between birth and postnatal day 21. There were no treatment-related effects on organ weights in adult rats. Decreases in absolute thymus gland and spleen weights (13–20%) and increases in relative brain weight (12–15%) in F₁ and F₂ pups at 300 mg/kg were likely to be related to reductions in body-weight gain observed in these animals (14–16%) and were not considered to be toxicologically significant.

There were no treatment-related pathology findings in adult F₀ and F₁ rats, including estrous cycles, sperm morphology and ovarian parameters. In F₁ pups at 300 mg/kg, there was a slight delay in vaginal opening (31.7, 32.1, 32.4, 33.3 days, control group to highest dose, respectively), which was statistically significant at 300 mg/kg, but this parameter was not assessed in F₂ pups. For each treatment group, and the controls, the day of vaginal opening ranged between day 29/30 and day 37. The principle difference in the pups at the highest dose was a reduction in the number of animals showing early vaginal opening (by day 29–31), rather than an increase in the number of animals with late vaginal opening (day 36/37). The birth weight of female pups at 300 mg/kg was unaffected in both generations, but a significantly lower body-weight gain was observed thereafter. Consequently, the slightly delayed vaginal opening was likely to reflect the delayed maturation of these pups rather than an in utero effect on sexual development. As some pyraclostrobin may have been excreted in maternal milk, pups begin to eat small amounts of the maternal ration well before weaning, and as the lower body weights developed only some days post partum, the effect was likely to reflect toxicity resulting from exposure post partum, and was not therefore considered to be a reproductive effect per se. An apparent increase in the incidence of sloped incisors in F₁ pups (on a pup and litter basis) at 75 and 300 mg/kg was not considered to be toxicologically significant since the effect was slight in nature, and values in the F₂ pups were similar to those in the control group for that generation.

The NOAEL for general and pup effects was 75 mg/kg (8.2 mg/kg bw per day) on the basis of reduced body-weight gain and food consumption in adults, reduced body-weight gain in pups, and a slight delay in vaginal opening in F₁ pups at 300 mg/kg. The NOAEL for reproductive effects was 300 mg/kg (33 mg/kg bw per day), the highest dose tested (Schilling et al., 1999b).

(b) *Developmental toxicity*

Groups of 25 female Wistar rats (aged 11–12 weeks) were given pyraclostrobin (purity, 98.9%) at a concentration of 0, 10, 25, or 50 mg/kg bw per day by gavage as a suspension in 0.5% aqueous Tylose solution on days 6–19 of gestation. Rats were housed in cages in controlled conditions and food and water were available ad libitum. All rats were checked at least once daily for mortality and clinical signs of toxicity and body weight and food consumption were recorded every 2–3 days until day 20 of gestation. After the final sacrifice, a corrected body-weight gain was calculated. On day 20 of gestation, all rats were sacrificed and examined macroscopically. The uterus and ovaries were removed and the weight of the “unopened” uterus, the number of corpora lutea and the number and distribution of implantation sites (classified as live fetuses or “dead implantations”) were recorded. Postimplantation losses were further classified as early or late resorptions or dead fetuses. Conception rate, pre- and postimplantation loss were recorded. At necropsy, each fetus was

Table 22. Reproductive performance and pup survival in a two-generation study in rats given diets containing pyraclostrobin

	Dietary concentration (mg/kg of feed)			
	0 (control)	25	75	300
<i>F₀ generation</i>				
Mean live pups/litter	14.2	13.4	12.3	12.9
Pup survival index (days 0–4)	93	87	100	93
Mean pup body weight (g)				
Males				
Postnatal day 1	6.6	6.5	6.7	6.8
Postnatal day 7	15.2	15.0	15.5	14.9
Postnatal day 21	52.8	53.7	52.8	47.4**
Females				
Postnatal day 1	6.2	6.2	6.5	6.4
Postnatal day 7	14.7	14.5	15.0	14.3
Postnatal day 21	51.4	51.3	50.3	45.2**
<i>F₁ generation</i>				
Mean No. of live pups delivered	98	99	99	99
Mean pup body weight (g)				
Males				
Postnatal day 1	6.6	6.6	6.7	6.5
Postnatal day 7	15.2	14.9	14.7	13.5**
Postnatal day 21	52.0	52.6	51.4	45.0**
Females				
Postnatal day 1	6.2	6.3	6.2	6.2
Postnatal day 7	14.5	14.6	14.2	13.1**
Postnatal day 21	49.8	50.2	48.9	43.5**

From Schilling et al. (1999b)
* $p \leq 0.05$; ** $p \leq 0.01$
† 1 dam died on the 1st day of lactation

Table 23. Organ weights and pathology findings for pups in a two-generation study in rats

	Dietary concentration (mg/kg of feed)							
	Control		25		75		300	
	M	F	M	F	M	F	M	F
<i>Organ weights</i>								
<i>F₁ pups:</i>								
Brain (g)	1.50	1.44	1.51	1.44	1.49	1.46	1.47	1.42
Spleen (g)	0.26	0.24	0.26	0.24	0.25	0.24	0.21**	0.21*
Thymus gland (g)	0.19	0.19	0.18	0.18	0.17*	0.18	0.16**	0.16**
Relative brain weight	2.73	2.85	2.79	2.88	2.85*	2.87	3.14**	3.14**
Relative spleen weight	0.46	0.47	0.47	0.47	0.47	0.47	0.45	0.46
Relative thymus gland weight	0.35	0.38	0.34	0.35	0.33	0.36	0.33	0.35
<i>F₂ pups:</i>								
Brain (g)	1.50	1.45	1.49	1.44	1.48	1.43	1.44**	1.39**
Spleen (g)	0.23	0.23	0.24	0.24	0.24	0.23	0.20	0.18**
Thymus gland (g)	0.17	0.18	0.18	0.18	0.17	0.17	0.14**	0.15**
Relative brain weight	2.88	2.92	2.84	2.85	2.88	2.91	3.20**	3.26**
Relative spleen weight	0.44	0.46	0.45	0.47	0.46	0.47	0.45	0.43
Relative thymus gland weight	0.33	0.35	0.33	0.36	0.33	0.35	0.31	0.35
<i>Pathology</i>								
F ₁ pups sloped incisors, Pups/litters	1/1		2/1		5/5		6/4	
F ₂ pups sloped incisors, Pups/litters	3/3		5/4		4/4		7/5	

From Schilling et al. (1999b)
F, Female; M, Male
* $p \leq 0.05$; ** $p \leq 0.01$

Table 24. Maternal findings in a study of developmental toxicity in rats given pyraclostrobin by gavage

Parameter	Dose (mg/kg bw per day)			
	0	10	25	50
Food consumption (g/animal per day), days 6–8	24.6	23.6	21.2**	17.9**
Body weight (g), day 20	369	373	354	350*
Body weight change (g), days 6–19	104	107	96	88**
Carcass weight (g)	290	290	277	272**
Corrected body-weight gain (g), from day 6 (g)	40.7	40.9	31.9*	22.3**

From Schilling et al. (1999c)

Dunnett *t*-test * $p < 0.05$; ** $p < 0.01$

Table 25. Summary of findings on litters and fetal parameters in a study of developmental toxicity in rats given pyraclostrobin by gavage

Finding	Dose (mg/kg bw per day)				Range for historical controls
	0 (control)	10	25	50	
Fetuses/litters examined	148/22	140/20	136/21	165/24	
Dilated renal pelvis					Mean, 16.7
Incidence	8 (6)	16 (10)	20 (9)	31 (15*)	
%	5.4 (27)	11 (50)	15 (43)	19 (63)	8.8–28.8 (30.4–79.2)
Fetuses/litters examined	158/22	151/20	147/21	178/25	
Incomplete ossification of sternebra					Mean, 24.6%
Incidence	29 (15)	39 (14)	43 (16)	61 (22)	
%	18 (68)	26 (70)	29 (76)	34 (88)	0–34.5% (95.7%)
Rudimentary cervical ribs					Mean, 2%
Incidence	1 (1)	2 (2)	2 (2)	9 (8*)	
%	0.6 (4.5)	1.3(10)	1.4 (9.5)	5.1 (32)	0.5–6.6% (4–27.3%)

From Schilling et al. (1999c)

Number of affected litters is shown in parentheses; * $p \leq 0.05$

weighed, sexed and examined macroscopically for external findings. The condition of the placentae, the umbilical cords, the fetal membranes and fluids were examined and individual placental weights were recorded. Approximately half of the fetuses from each litter were examined for visceral abnormalities, whilst the remainder were examined for skeletal abnormalities.

There were no deaths, no treatment-related clinical signs, no effects on litter or fetal parameters, and pathology in adults was unaffected. Food consumption was consistently and significantly reduced at 25 and 50 mg/kg bw per day from immediately after initiation of dosing until sacrifice, and the magnitude of the effect was dose-related for all except day 13 when the values were essentially the same for these two groups. At 50 mg/kg bw per day, body-weight gain was reduced by 16% and food consumption by 11% between gestation days 6 and 19. Corrected body-weight gain (i.e. minus the gravid uterus) was reduced by 22 and 45% at 25 and 50 mg/kg bw per day (40.7, 40.9, 31.9, 22.3 g, control group to highest dose, respectively) and carcass weights by 6% at 50 mg/kg bw per day. Gravid uterus weights were unaffected by treatment.

The incidences of dilated renal pelvis, incomplete ossification of sternebra and rudimentary cervical ribs were increased on a fetal and litter basis at 50 mg/kg bw per day. Although the incidence of these effects were within the range of historical control data, they

are concluded to be related to treatment because there was a clear dose–response relationship and/or they were at the upper limit of the range for historical controls. The NOAEL for maternal effects was 10 mg/kg bw per day on the basis of reduced body-weight gains and food consumption at 25 mg/kg bw per day. The NOAEL for developmental effects was 25 mg/kg bw per day on the basis of an increased incidence of dilated renal pelves, incomplete ossification of sternebra and rudimentary cervical ribs at 50 mg/kg bw per day (Schilling et al., 1999c).

Rabbits

Groups of 24 or 25 pregnant Himalayan rabbits (aged 24–29 weeks) were given pyraclostrobin (purity, 98.9%) at a concentration of 0, 5, 10 and 20 mg/kg bw per day as a suspension in 0.5% aqueous Tylose solution by gavage on days 7–28 of gestation. Rabbits were housed in cages in controlled conditions and food and water were available ad libitum. All rabbits were checked at least once daily for mortality and clinical signs of toxicity; body weight was recorded every 2–3 days during gestation and food consumption was recorded daily. After the final sacrifice, a corrected body-weight gain was calculated. On day 29 of gestation, all surviving rabbits were sacrificed and examined macroscopically. The uterus and ovaries were removed and the weight of the unopened uterus, the number of corpora lutea and the number and distribution of implantation sites (classified as live fetuses or dead implantations) were recorded. Dead implantations were further classified as early or late resorptions or dead fetuses. Conception rate, pre-implantation and postimplantation losses were recorded. At necropsy, each fetus was weighed and examined macroscopically for external findings. The condition of the placentae, the umbilical cords, the fetal membranes and fluids were examined and individual placental weights were recorded. The sex of the fetuses was determined by internal examination of the gonads. Approximately half of the fetuses from each litter were examined for visceral abnormalities, whilst the remainder were examined for skeletal abnormalities.

One control and one dam at 10 mg/kg bw per day died during or shortly after dosing on days 9 and 25 of gestation, respectively. At 10 and 20 mg/kg bw per day, blood was observed in the bedding of two and four does respectively between days 16 and 29 of gestation and was probably secondary to the increased resorption rate seen at these doses. A marked, clearly dose-related, but transient reduction in food consumption (by 31–77%) and body-weight gain (by 13–25%) was observed immediately after initiating dosing. Both food consumption and weight gain subsequently tracked rapidly towards control levels and were equal to those of the control group by day 4 after dosing for the groups at 5 and 10 mg/kg bw per day and by about day 5 for the group at 10 mg/kg bw per day. Despite continued treatment for a further 17 days, both food consumption and weight gain were comparable to that in the control group throughout the remainder of the study. This pattern of weight gain and food consumption variation is consistent with a transient food aversion related to local gastrointestinal tract and/or sensory disturbance caused by pyraclostrobin administered by gavage. Whilst this effect is clearly treatment- and dose-related, it is likely to be dependent on the local concentration of the test material administered, has little relevance to human dietary risk assessment and was consequently disregarded by the present reviewer for the purpose of considering an acute RfD; however, this does prevent the identification of a NOAEL for maternal toxicity in this study. The marked reduction in food intake is likely to have reduced the nutritional status of the does at a critical point in pregnancy, around the time of implantation, and may have contributed to the increased postimplantation loss observed at 10 and 20 mg/kg bw per day and possibly also the increased incidence

of missing lumbar vertebrae. This conclusion is supported to some extent by studies showing similar effects on the fetuses of rabbits and rats following feed restriction, although the duration of the feed restriction was longer than that observed in this study. A 25% feed restriction in rabbits, for example, significantly reduced the number of fetuses per pregnant female (Warren & Kirkpatrick, 1978). In pregnant rabbits fed between 15 and 50% of the control dietary intake from day 6 to day 20 of gestation, fetal weights were reduced and the incidence of cleft palate and fused sternebrae were markedly increased at 15% of the maternal diet and to a lesser extent at 25% of the maternal diet (Noda et al., 1993). Similarly, in rats fed <80% of the control dietary intake throughout gestation, an increased number of resorptions and small fetuses, decreased fetus weights and retarded bone development were observed (Waalkens-Berendsen et al., 1990).

Defaecation was reduced in one and 10 does between days 10 and 14 of gestation at 10 and 20 mg/kgbw per day respectively and is likely to be secondary to reduced food intake. At termination, net maternal carcass weights (body weight minus the conceptus) of treated dams were comparable to those of controls (2608, 2504, 2580, 2539 g, control group to highest dose, respectively) and net body-weight gains/losses were also similar across groups (-136, -142, -133, -147). There were no fetal external or visceral abnormalities attributable to treatment and no treatment-related pathology findings in adults. A slight, non significant, increase in the incidence of skeletal malformations at 20 mg/kgbw per day was driven by an increase in the incidence of absent lumbar vertebrae at this dose (fetal incidence of 0.6, 0.7, 0.8 and 3.7%, control group to highest dose, respectively) which exceeded the mean (0.3%) and range (0–0.9) for historical controls and is therefore concluded to be treatment-related despite the lack of statistical significance. At 10 and 20 mg/kgbw per day, two and three does, respectively, had no live fetuses. At 10 and 20 mg/kgbw per day, total resorptions were increased in comparison to control (6.2, 10.2, 17.8, 38.6% control group to highest dose) primarily owing to increased early resorptions. Although the postimplantation loss that occurred at 10 mg/kgbw per day was within the range of data for historical controls (range, 5.2–20.1%; mean, 9.9%), it was concluded to be treatment-related both by the study author and the present reviewer on the basis of a comparison with the concurrent control group, association with total litter losses and blood in the bedding, and on the clear dose-dependency. Gravid uterus weight was reduced by 23 and 41% at 10 and 20 mg/kgbw per day, as was fetal weight. A NOAEL for maternal toxicity was not identified owing to a marked decrease in weight gain and food consumption at all doses. The NOAEL for fetal effects was 5 mg/kgbw per day on the basis of increased postimplantation losses and reduced fetal weight at ≥ 10 mg/kgbw per day. For the reasons discussed, undue weight was not attached to the absence of a lowest-observed-effect level (LOEL) for maternal toxicity in the presence of fetal resorptions, other than the markedly reduced body-weight gain, owing to the conclusion that the nutritional status of the does was likely to have been compromised by the marked transient reduction in food intake (Schilling et al., 1999d).

Groups of 25 presumed pregnant Himalayan rabbits (Chbb:HM,; weight, approximately 2570 g; from Boehringer Ingelheim Pharma KG, Germany) were given pyraclostrobin at a dose of 0, 1, 3, or 5 mg/kgbw per day by gavage on days 7–28 of gestation. The animals were observed twice daily for mortalities and once per day for clinical signs of toxicity. Body weights were measured every 2–3 days and food consumption was measured daily. The animals were killed on day 29 of gestation and the number of corpora lutea, implantations, early and late resorptions, and viable and non-viable fetuses were evaluated. At necropsy of dams, gross pathological examination was conducted and the gravid uterus, fetuses and placenta were weighed. Carcass weight (terminal body weight minus gravid

Table 26. Maternal findings in a study of developmental toxicity in rabbits

Parameter	Dose (mg/kgbw per day)			
	0 (control)	5	10	20
Food consumption; days 7–8 (g/animal/day)	98.1	35.7**	20.4**	10.5**
Food consumption; days 10–11 (g/animal/day)	96.4	90.4	74.8**	35.1**
Food consumption; days 13–14 (g/animal/day)	90.9	84.0	87.6	73.2*
Food consumption; days 14–15 (g/animal/day)	86.9	80.2	83.5	79.2
Body weight; day 29 (g)	2961	2807	2851	2748**
Body-weight change; days 7–9 (g)	–3.8	–43.8**	–85.5**	–146.3**
Body-weight change; days 9–11 (g)	0.2	14.8	21.6	–24.2
Body-weight change; days 11–14 (g)	23.4	15.7	25.8	–46.8*
Body-weight change; days 14–16 (g)	52.1	44.5	42.7	54.2
Gravid uterus (g)	352.6	302.6	271.2*	209.6**
Corrected body-weight gain; from day 7 (g)	–135.7	–142.4	–132.9	–146.8

From Schilling et al. (1999d)
Dunnett *t*-test * *p* < 0.05; ** *p* < 0.01

Table 27. Summary of litter and fetal parameters in a study of developmental toxicity in rabbits

Parameter	Dose (mg/kgbw per day)			
	0 (control)	5	10	20
No. of does with live young	24	24	20	22
Mean No. of live young	6.9	6.0	6.2	4.9**
Mean No. of corpora lutea	8.0	7.9	7.8	7.7
Mean No. of implantations	7.4	6.6	6.9	7.0
Implantation loss (%)				
Pre-implantation	7.0	15.7	10.6	10.1
Postimplantation	6.2	10.2	17.8	38.6**
Mean no. of resorptions				
Early	0.42	0.54	1.2	2.6**
Late	0.04	0.04	0.1	0.1
Early and late	0.5	0.6	1.3	2.7**
Placental weight (g)	4.5	4.3	4.1	4.2
Fetal body weight (g)	37.0	37.0	35.2	35.1
Sex ratio (% males)	50.0	53.1	52.8	52.3
Gravid uterus (g)	352.6	302.6	271.2*	209.6**
<i>Incidence of malformations and variations</i>				
Malformations (% of fetuses)				
External	0.6	0.7	1.6	0
Visceral	0.6	2.1	4.9	0.9
Skeletal	3.6	2.8	4.1	8.4
Variations (% of fetuses)				
External	0.6	0.7	1.6	0
Visceral	11	2.8	9.8	6.5
Skeletal	64	62	67	66

From Schilling et al. (1999d)
* *p* ≤ 0.05; ** *p* ≤ 0.01

uterus weight) and corrected body-weight gain (carcass weight minus day-7 body weight) were calculated.

There were no deaths or clinical signs of toxicity in any of the treated dams. There were no effects of treatment on implantation, postimplantation loss or the number of live fetuses, no effects on gravid uterus, placenta or fetal weights and carcass weight was similar in all groups. Food consumption was reduced immediately after initiation of dosing at 3 (25%) and 5 mg/kgbw per day (40%), but it recovered during the remainder of the dosing

period. The transient reduction in food consumption observed just after dosing resulted in food consumption being 15% and 20% lower across the entire dosing period at 3 and 5 mg/kg bw per day respectively, but the changes were not statistically significant. Body-weight loss was observed in all groups, including the control group before dosing began. Body-weight loss (between about 0.1 and 0.5% of body weight) also occurred immediately after dosing at 3 and 5 mg/kg bw per day, but body-weight gain had recovered to be similar in all groups before the day 4 of dosing and generally remained similar in all groups until the end of the dosing period. When results from days 7–28 were considered together, body-weight gains showed no effect of treatment at doses <5 mg/kg bw per day. Although there was an apparent dose-related decrease in mean corrected body-weight gains (–83, –103, –109, –137 g, control group to highest dose, respectively), there was considerable individual variation in all groups, with the standard deviation varying between 90 and 113 and therefore no statistically significant change was observed. The NOAEL for maternal toxicity was 3 mg/kg bw per day on the basis of reduced food consumption and body weight gain at 5 mg/kg bw per day. There were no effects on the fetuses that were considered to be treatment-related and therefore the NOAEL for developmental toxicity was 5 mg/kg bw per day, the highest dose tested (Schilling, Hellwig & van Ravenzwaay, 2001).

2.6 *Special studies*

(a) *Neurotoxicity*

The neurotoxicity of a single dose of pyraclostrobin (purity, 99%) at 0, 100, 300 and 1000 mg/kg bw administered by gavage in 0.5% aqueous carboxymethylcellulose was assessed in groups of 10 male and 10 female Wistar rats (aged 49 days). Dose selection was based on the results of a range-finding study in which three rats of each sex were given pyraclostrobin at a dose of 1000 and 2000 mg/kg bw per day by gavage (clinical examinations were performed outside the home cage immediately after dosing as well as at 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h and 1, 2 and 3 days after dosing. Animals were individually housed in cages in controlled conditions and food and water were available ad libitum. Clinical signs of toxicity were assessed at least once daily and body weight was assessed weekly. Functional observational batteries (FOB) and motor activity were assessed 7 days before dosing, and on days 0 (within a few hours after dosing), 7 and 14. The FOB consisted of four parts, starting with passive observations (without disturbing the animals), followed by removing the animal from its home cage and “open field observations” in a standard arena. Two weeks after dosing, five rats of each sex per dose were sacrificed by “in situ perfusion fixation” and were subjected to neuropathology examinations, including an examination for gross lesions. The remaining five rats of each sex per group were sacrificed without further examination. Dorsal and ventral root fibres and associated dorsal root ganglia were collected from the C3–C6 and L1–L4 areas of the spinal cord and the proximal sciatic nerve and tibial and sural nerves (at the knee) were examined in animals in the control group and at 1000 mg/kg bw per day. The brain (frontal lobe, parietal lobe with diencephalon, midbrain with occipital and temporal lobe, pons, cerebellum), spinal cord (cross sections of cervical swelling (C3–C6) and lumbar swelling (L1–L4)) and peripheral nervous system (Gasserian ganglia with nerve and gastrocnemius muscle) were examined microscopically in animals in the control group and at 1000 mg/kg bw per day.

There were no deaths in either the range-finding or main studies. In the range-finding study, animals at 1000 and 2000 mg/kg bw per day had diarrhoea and were apathetic on the first day only, and piloerection occurred in animals at 2000 mg/kg bw per day, lasting until day 6. In the main study, diarrhoea occurred at 300 (two males and one female) and 1000

(five males and four females) mg/kg bw per day and piloerection was observed in four females at 1000 mg/kg bw per day, resolving by day 7. Body-weight gain was reduced by 33% in males at 1000 mg/kg bw per day during the first week of the study. There were no treatment-related effects in the FOB or motor activity parameters on days 7 and 14, and no neuropathological effects were observed (Mellert et al., 1999h).

Groups of 10 male and female Wistar rats (aged 49 days) were given diets containing pyraclostrobin (purity, 97.1%) at a concentration of 0, 50, 250, or 750 mg/kg of feed (males only), equal to 0, 4, 17 and 50 mg/kg bw per day, and 1500 mg/kg (equal to 110 mg/kg bw per day, females only) for 3 months. Animals were individually housed in cages in controlled conditions, and food and water were available *ad libitum*. Clinical signs of toxicity were assessed at least once daily and body weight, food and water consumption and food conversion efficiency $[(\text{body-weight gain})/(\text{food consumed}) \times 100]$ were assessed weekly. FOB and motor activity were assessed 7 days before dosing, and on days 22, 50 and 85. The FOBs consisted of four parts, starting with passive observations (without disturbing the animals), followed by removing the animal from its home cage and “open field observations” in a standard arena. At the end of the treatment period, five rats of each sex per dose were sacrificed by *in situ* perfusion fixation for neuropathology examinations, including an examination of other tissues for gross lesions. The remaining five rats of each sex per group were sacrificed without further examination. The brain weight (without the olfactory bulb) was recorded from all groups, prior to its preparation for microscopic examination. Dorsal and ventral root fibres and associated dorsal root ganglia were collected from the C3–C6 and L1–L4 areas of the spinal cord and the proximal sciatic nerve and tibial and sural nerves (at the knee) were examined in animals in the control group and in males at 750 mg/kg and females at 1500 mg/kg. The brain (frontal lobe, parietal lobe with diencephalon, midbrain with occipital and temporal lobe, pons, cerebellum), spinal cord (cross sections of cervical swelling (C3–C6) and lumbar swelling (L1–L4)) and peripheral nervous system (Gasserian ganglia with nerve and gastrocnemius muscle) were examined microscopically in animals in the control group and in males at 750 mg/kg and in females at 1500 mg/kg.

There were no deaths, treatment-related clinical signs or effects on motor activity or neuropathology findings. In males at 750 mg/kg and females at 1500 mg/kg, reduction were seen in body-weight gain by approximately 18%, food consumption by up to 40% and water consumption by up to 28%. Grip strength of the forelimbs was impaired by 17% in females at 1500 mg/kg on day 85. Impaired grip strength is a non-specific sign and, in isolation, does not indicate selective neurotoxicity. Consequently, the NOAEL for neurotoxicity in this study was 750 mg/kg (approximately 50 mg/kg bw per day), the highest dose tested in males. The overall NOAEL was 250 mg/kg (17 mg/kg bw per day) on the basis of reduced body-weight gain, food consumption and water consumption at the dose above (750 mg/kg in males and 1500 mg/kg in females) (Mellert et al., 1999i).

(b) *Mechanistic studies*

Reduced serum alanine aminotransferase and/or alkaline phosphatase activity in rats has been observed both for pyraclostrobin and for other strobilurins, such as kresoxim-methyl, for example (Annex 1, reference 85).

A series of experiments were conducted to investigate the cause of the observed decrease in serum alkaline phosphatase and alanine aminotransferase activities in rats. In the first study, blood was taken from five male and five female rats fed with a normal diet,

before administration of diet containing pyraclostrobin at a concentration of 8000 mg/kg (equal to 400 mg/kgbw per day) for about 2 weeks. At the end of this period, blood was again taken from these animals and sera were analysed both for total alkaline phosphatase and for isozyme composition. In the second experiment, the effect of dietary manipulation on the enzyme and isozyme activities of female rats treated with pyraclostrobin and of controls was investigated.

Total alkaline phosphatase activities in treated animals fell to approximately 66% of pre-treatment levels. This fall in alkaline phosphatase activities was due almost entirely to a decline in the intestinal isozyme, with that derived from rat liver and bone being unaffected. In rats, the activity of intestinal alkaline phosphatase in serum is strongly influenced by ingestion of fats and this was confirmed in the dietary manipulation study where addition of olive oil in the diet increased intestinal alkaline phosphatase activities by about 50% in both control rats and in rats treated with pyraclostrobin. Although the rats treated with pyraclostrobin on the diets supplemented with olive oil had lower alkaline phosphatase activities than before the commencement of treatment with pyraclostrobin, the levels did not fall to below that seen in control rats on a normal diet. Thus, addition of olive oil to the diet increased total alkaline phosphatase activities in both treated and control animals, through an increase in the activity of the intestinal isozyme of alkaline phosphatase. Addition of sucrose to the diet did not significantly affect alkaline phosphatase activities in either control animals or animals treated with pyraclostrobin. Similarly, the activity of intestinal alkaline phosphatase in fasted, untreated, animals was 75% lower than that in fed animals. Both total and intestinal alkaline phosphatase activities in fasted animals treated with pyraclostrobin were similar to those in untreated fasted animals (intestinal isozyme activity, 33 U/l compared with 40 U/l for the fasted control animals). Consequently, the greatest effect on the activity of alkaline phosphatase in rat serum was due to the fasting state of the animals.

In humans, little intestinal alkaline phosphatase is present in the serum owing to active scavenging by the liver; consequently, pyraclostrobin would be unlikely to produce a decline in serum alkaline phosphatase activity if ingested by humans.

Alanine aminotransferase activity in fasted rats was lower by approximately 30–40% in both control rats and in rats treated with pyraclostrobin compared with normally fed rats. Supplementation of normal diets with sucrose or olive oil had little effect on alanine aminotransferase activities in untreated rats, but appeared to reduce the effect of pyraclostrobin on the activity of this enzyme. The addition of pyridoxyl-5'-phosphate to the assay mixture had a small but constant stimulating effect on alanine aminotransferase activity in both control rats and rats treated with pyraclostrobin, indicating that depletion of, or interference with, this co-factor is not involved in the mechanism of the dietary or pyraclostrobin effect on alanine aminotransferase. The study author concluded that the effects of pyraclostrobin on alkaline phosphatase and alanine aminotransferase activities in rats were secondary to the altered dietary status of these animals (Moss, 1994).

(c) *Comparison with other members of the strobilurin class of fungicides*

On the basis of publicly available toxicology summaries for kresoxim-methyl, azoxystrobin and trifloxystrobin¹, in conjunction with the studies on pyraclostrobin reviewed in

¹ Published on the Internet by, for example, the Australian and USA pesticide regulatory bodies, among others. See www.apvma.gov.au (public release summaries) and www.epa.gov/fedregister/ (pesticide tolerance notices) for examples.

this monograph, the toxicological profile of pyraclostrobin is largely consistent with that of the strobilurin fungicides as a chemical class. Compounds in this class of fungicides are not reproductive or developmental toxins, are not genotoxic and, with the exception of kresoxin-methyl at very high doses in rats, are not carcinogenic.

Comments

In rats treated orally with radiolabelled pyraclostrobin, about 50% of the administered dose was absorbed. Concentrations of radiolabel in the blood peaked initially after 30 min, followed by a secondary peak at 8 h or 24 h. Most (74–91%) of the radiolabelled dose was eliminated in the faeces, with the remainder (10–13%) in the urine. The pattern of excretion was not affected by repeated administration. In rats, the metabolism of pyraclostrobin proceeds through three main pathways. The methoxy group on the tolyl-methoxycarbamate moiety is readily lost, with few major metabolites retaining this group. Hydroxylation of the benzene and/or pyrazole rings is followed by conjugation with glucuronide and, to a lesser extent, sulfate. Many metabolites are derived from the chlorophenol-pyrazole or tolyl-methoxycarbamate moieties of pyraclostrobin after cleavage of the ether linkage between these two groups, with subsequent ring hydroxylation and glucuronide or sulfate conjugation. Metabolites were similar in both sexes and at all doses. No unchanged parent compound was found in the bile or urine and only small amounts were found in the faeces. Most of the radiolabel isolated from kidney tissues was in the form of the unchanged parent compound and a demethoxylated derivative.

Pyraclostrobin has low acute toxicity when administered orally or dermally, with LD₅₀s of >5000 and >2000 mg/kg bw, respectively, and no deaths in either case. The compound has moderate toxicity when administered by inhalation, with an LC₅₀ of 0.31–1.07 mg/l when acetone is used as the solvent, and 4.07–7.3 mg/l when Solvesso is used as the solvent.

Pyraclostrobin is a mild dermal and ocular irritant, but is not a skin sensitizer. Clinical signs after oral administration consisted of dyspnoea, staggering, piloerection, and diarrhoea in all animals, which resolved by day 6. There were no pathology findings.

In short-term studies in mice, rats and dogs, the major toxicological findings after repeated doses of pyraclostrobin involved duodenal mucosal hypertrophy and, in some studies in rodents, erosion/ulceration of the stomach mucosa. These findings are suggestive of a local irritant action, a conclusion that is supported by the occurrence of vomiting in dogs. However, pyraclostrobin is not a severe dermal irritant, although in rabbits the irritation was somewhat prolonged.

Reductions in body-weight gain and food consumption were observed in all species, although the pattern of the response and relationship to treatment varied. To some extent, these effects suggest local disturbance to the gastrointestinal tract and taste aversion, particularly in rabbits, although a systemic effect may also be involved, especially in rodents.

In short-term studies with repeated doses of pyraclostrobin, reduced body-weight gains were accompanied by reductions in clinical chemistry parameters (including concentrations of total protein, globulin, glucose, triglycerides and creatinine) and reduced fat storage in the liver. These observations may be secondary to a disturbance of normal meta-

bolic processes following the disruption of mitochondrial respiration, the primary biochemical mechanism by which pyraclostrobin acts. These effects may also reflect a reduced nutritional status caused by reduced food intake or food conversion. Reduced body-weight gain largely determined the minimally toxic dose in lifetime studies in rats and mice, but was not associated with toxicologically relevant alterations in clinical pathology values where these were measured in rats.

Mild anaemia associated with extramedullary haematopoiesis in the spleen was observed in rodents fed with repeated doses of approximately ≥ 400 mg/kg (equal to 120 mg/kg bw per day in mice and 42 mg/kg bw per day in rats) in short-term studies. Hepatocellular hypertrophy, in the absence of significant alterations in relevant clinical chemistry parameters or other histological evidence of liver injury, was also observed in rats at 120 mg/kg bw per day.

Pyraclostrobin gave negative results in an adequate battery of studies of genotoxicity in vitro and in an assay for micronucleus formation in bone-marrow cells of mice in vivo.

The Meeting concluded that pyraclostrobin was unlikely to be genotoxic.

The carcinogenic potential of pyraclostrobin was studied in rats and mice. While the incidence of hepatocellular adenomas was slightly increased in one study in rats fed with pyraclostrobin at 200 mg/kg (equal to 9 mg/kg bw per day), no increase was observed in a concurrent lifetime study. Moreover, the incidence of liver adenomas in controls was considerably higher (20% versus 8%), suggesting that a low value for controls contributed to the apparent effect observed in the first study. There was no evidence of carcinogenic potential in mice and rats. This conclusion is supported by the observation that other strobilurin fungicides have not shown carcinogenic activity of relevance to human risk assessment.

On the basis of the above consideration and the absence of genotoxicity, the Meeting concluded that pyraclostrobin is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproductive toxicity in rats, body-weight gains and food consumption were reduced in adults and lower body-weight gains and slightly delayed vaginal patency were observed in pups at a dose of 300 mg/kg (equal to 33 mg/kg bw per day). The NOAEL for general and pup toxicity was 75 mg/kg (equal to 8.2 mg/kg bw per day). The NOAEL for effects on reproductive performance was 300 mg/kg, the highest dose tested.

Two studies of developmental toxicity were conducted in rabbits and one in rats. Maternal toxicity consisting of reduced body-weight gains and food consumption was observed at ≥ 25 mg/kg bw per day in rats, and at ≥ 5 mg/kg bw per day in rabbits. In rats, the reduction in food consumption persisted beyond the treatment period and the corrected body-weight gains at termination were also reduced. In rabbits, a transient but marked reduction in food intake (and consequently in body-weight gain) after initiation of dosing was observed which resolved within 3–5 days, despite continued dosing. The pattern of the reduced body-weight gains and food consumption in rabbits indicated that they are likely to be caused by local effects on the gastrointestinal tract related to high concentrations of pyraclostrobin or taste disturbance resulting from regurgitation or leakage of the gavaging solution. Consequently, the Meeting concluded that these effects did not reflect systemic toxicity caused by pyraclostrobin. Nonetheless, the reduced nutritional status of dams,

which was caused by lower food intakes at a critical time in gestation at and immediately after implantation, must be taken into account when considering the significance of the observed fetal effects at doses that were not otherwise maternally toxic. The NOAEL for maternal toxicity was 10mg/kg bw per day in rats and 3 mg/kg bw per day in rabbits.

Pyraclostrobin was not teratogenic in rats, but fetal effects consisting primarily of developmental delay (incomplete ossification of sternebra and rudimentary cervical ribs) and an increased incidence of dilated renal pelvises, were observed at a dose of 50mg/kg bw per day. In rabbits, fetal effects consisting of increased postimplantation losses were observed at ≥ 10 mg/kg bw per day. A slight, non-significant increase in the incidence of skeletal malformations observed at 20mg/kg bw per day was driven by an increase in the incidence of absent lumbar vertebrae at this dose. Although the incidence was not statistically significant, it exceeded the mean for historical control values and the upper bound of the range, and the Meeting could not exclude the possibility that the effect was potentially treatment-related. The effects seen in rabbit fetuses were likely to be secondary to the marked nutritional deficit in the dams at a critical time in gestation. The Meeting concluded, however, that the available data did not provide a sufficient basis on which to confidently exclude other potential mechanisms; consequently, the NOAEL for developmental toxicity in the study in rabbits was 5 mg/kg bw per day on the basis of these fetal effects. The developmental NOAEL was 25 mg/kg bw per day in rats.

Pyraclostrobin was investigated for neurotoxicity in rats in a study in which a single dose was administered by gavage and in a 90-day study of pyraclostrobin in the diet. The NOAELs for neurotoxicity were 2000mg/kg bw and 750mg/kg (equal to 50mg/kg bw per day) respectively, the highest doses tested. Pyraclostrobin was not found to be neurotoxic.

The Meeting concluded that the existing database was adequate to characterize the potential hazard of pyraclostrobin to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg bw based on a NOAEL of 3.4mg/kg bw per day identified in two 2-year studies in rats, on the basis of reduced body-weight gain and altered liver and stomach histology at 200 mg/kg and using a 100-fold safety factor.

Pyraclostrobin is not acutely toxic and short-term dosing produced no significant general toxicity; however, fetal resorptions were increased at a dose of 10mg/kg bw per day in a study of developmental toxicity in rabbits. Although a transient but marked reduction in food intake, and consequently in body-weight gain, was observed at doses of 5 mg/kg bw per day and above after initiation of dosing in studies of developmental toxicity in rabbits, this effect resolved within 3–5 days, despite continued dosing. The pattern of the observations indicates they are likely to be caused by local gastrointestinal tract effects related to high concentrations of pyraclostrobin, or to taste disturbance resulting from regurgitation or leakage of the gavaging solution. Consequently, the Meeting concluded that these observations did not reflect systemic toxicity caused by pyraclostrobin and were not used to establish the acute RfD. The Meeting established an acute RfD of 0.05 mg/kg bw, based on the NOAEL of 5 mg/kg bw per day for fetal toxicity at 10mg/kg bw per day in the study of

developmental toxicity in rabbits and using a 100-fold safety factor. Further information on the relationship between irritation of the gastrointestinal tract and reduced body-weight

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of toxicity and carcinogenicity ^a	Toxicity	30 mg/kg, equal to 4.1 mg/kg bw per day	120 mg/kg, equal to 17 mg/kg bw per day
		Carcinogenicity	120 mg/kg, equal to 17 mg/kg bw per day ^b	—
Rat	2-year study of toxicity and carcinogenicity ^a	Toxicity	75 mg/kg, equal to 3.4 mg/kg bw per day	200 mg/kg, equal to 9 mg/kg bw per day
		Carcinogenicity	200 mg/kg, equal to 9 mg/kg bw per day ^b	—
	3-month study of neurotoxicity ^a	Neurotoxicity	750 mg/kg, equal to 50 mg/kg bw per day ^b	—
		Toxicity	250 mg/kg equal to 17 mg/kg bw per day	750 mg/kg, equal to 50 mg/kg bw per day
	Two-generation study of reproductive toxicity ^a	Parental and offspring toxicity	75 mg/kg, equal to 8.2 mg/kg bw per day	300 mg/kg, equal to 33 mg/kg bw per day
	Study of developmental toxicity ^c	Maternal toxicity	10 mg/kg bw per day	25 mg/kg bw per day
		Embryo- and fetotoxicity	25 mg/kg bw per day	50 mg/kg bw per day
Rabbit	Study of developmental toxicity ^c	Maternal toxicity	3 mg/kg bw per day	5 mg/kg bw per day ^d
		Embryo- and fetotoxicity	5 mg/kg bw per day	10 mg/kg bw per day
Dog	1-year study of toxicity ^a	Toxicity	200 mg/kg, equal to 5.4 mg/kg bw per day	400 mg/kg, equal to 11 mg/kg bw per day

^aDiet

^bHighest dose tested

^cGavage

^dA marked but transient reduction in maternal food intake occurred immediately after initiation of dosing at higher concentrations

gains in pregnant rabbits, and the effect of maternal nutritional deficit on fetal resorptions, may allow the acute RfD to be refined.

Estimate of acceptable daily intake for humans

0–0.03 mg/kg bw

Estimate of acute reference dose

0.05 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

- Observations in humans
- Studies in rabbits to explore the relationship between a marked reduction in food intake at the start of pregnancy and fetal survival and development

Summary of critical end-points for pyraclostrobin

Absorption, distribution, excretion and metabolism in animals

Rate and extent of oral absorption	Rapid, approximately 50%
Dermal absorption	1.6–2.6% in rats in vivo, 3–8% across human skin in vitro (from an unspecified formulation)
Distribution	Rapidly and widely distributed with highest concentrations in the gastrointestinal tract, liver and kidneys
Rate and extent of excretion	Largely complete within 48 h; approximately 15% in urine and 85% in the faeces; 35–40% of the dose was excreted in the bile
Potential for accumulation	No evidence of significant accumulation
Metabolism in mammals	Extensively metabolized with subsequent glucuronide and sulfate conjugation; the metabolites are unlikely to be toxicologically significant
	No unchanged parent compound in the bile or urine and only small amounts in the faeces.
Toxicologically significant compounds (animals, plants and the environment)	Parent compound

Acute toxicity

Rat, LD ₅₀ , oral	>5000 mg/kg bw (no deaths)
Rat, LD ₅₀ , dermal	>2000 mg/kg bw (no deaths)
Rat, LC ₅₀ inhalation	0.310–1.070 mg/l (4 h exposure, head and nose only) in acetone 4.07–7.3 mg/l (4 h exposure, head and nose only) in Solvesso solvent
Rabbit, dermal irritation	Slight but prolonged
Rabbit, ocular irritation	Slight
Skin sensitization	Not sensitizing (Magnusson & Kligman)

Short-term studies of toxicity

Target/critical effect	Ulceration of the glandular stomach in mice, hypertrophy of the duodenal mucosa in mice, rats and dogs, vomiting and diarrhoea in dogs, anaemia in mice and rats, decreased body-weight gains in mice, rats and dogs, hepatocellular hypertrophy in rats
Lowest relevant oral NOAEL	4 mg/kg bw per day (mice)
Lowest relevant dermal NOAEL	100 mg/kg bw per day (rats)
Lowest relevant inhalational NOAEC	No data

Genotoxicity

No genotoxic potential

Long-term toxicity and carcinogenicity

Target/critical effect	Reduced body-weight gains in rats and mice, elevated liver weights in mice, altered liver and stomach histology in rats
Lowest relevant NOAEL	3.4 mg/kg bw per day (two 2-year studies in rats)
Carcinogenicity	Not carcinogenic in rats or mice

Reproductive toxicity

Reproductive target/critical effect	None
Lowest relevant reproductive NOAEL	>33 mg/kg bw per day (two-generation study in rats)
Developmental target/critical effect	Increased postimplantation losses and reduced fetal weight
Lowest relevant developmental NOAEL	5 mg/kg bw per day (rabbits)
Neurotoxicity/delayed neurotoxicity	No evidence of neurotoxicity in a 3-month study in rats at doses of up to 50 mg/kg bw per day

Medical data

No adverse effects have been reported but the data are limited as pyraclostrobin is a new substance

Summary	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Rat, 2-year	100
Acute RfD	0.05 mg/kg bw	Rabbit, developmental	100

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PYRETHRINS (addendum)

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Explanation

Pyrethrins (pyrethrum extracts) derived from flowers of chrysanthemum of the genus *Chrysanthemum* have been used as insecticides for a long time. Pyrethrum, the active principle containing pyrethrin isomers, was evaluated toxicologically by the JMPR in 1965, 1966, 1967, 1968, 1969, 1970 and 1972. In 1999, the compound was re-evaluated on the basis of new studies that used a blend of refined pyrethrum extracts from plants in four major growing areas, with a total pyrethrin content of 57.6%. The 1999 JMPR established an acceptable daily intake (ADI) of 0–0.04mg/kgbw on the basis of the no-observed-adverse-effect level (NOAEL) for liver effects in a new 2-year study in rats, and a safety factor of 100. At the same Meeting, an acute reference dose (RfD) of 0.2mg/kgbw was established on the basis of the NOAEL in a study of acute neurotoxicity in rats, and a safety factor of 100.

The 1999 JMPR concluded that increased incidences of liver and thyroid tumours observed in rats treated with pyrethrins are threshold phenomena of negligible relevance to the low doses to which humans are exposed. In order to confirm this, the Meeting recommended that additional studies be performed to investigate the mechanism by which pyrethrins cause tumorigenesis in the liver and thyroid. The 1999 Meeting also suggested that a test for gene mutation in mammalian cells and more detailed information on case reports of adverse health effects in humans, for which only an abstract was available, should be submitted for evaluation.

The following information was made available to the present Meeting: a new test for gene mutation in mammalian cells, the full report of the mechanistic studies on liver and thyroid tumorigenesis in rats; and the full report of the analysis of case reports of human exposures to consumer products containing pyrethrins and/or pyrethroids.

Evaluation for acceptable daily intake and acute reference dose

1. Genotoxicity

In a study that complied with good laboratory practice (GLP), pyrethrins did not induce gene mutations at the thymidine kinase (*Tk*) locus in mouse lymphoma L5178Y cells (see Table 1). The first assay, conducted in the presence of metabolic activation, was not valid since the frequency of mutation was not increased for the positive control. In the second assay, the frequency of mutation was significantly increased with pyrethrin at a concentration of 85 and 52 µg/ml and was equivocally increased at 61 and 26 µg/ml. In the third assay with duplicates of pyrethrins at a concentration of 72, 61, 52, 26 and 13 µg/ml, no significant increase in the frequency of mutation was observed at any concentration (Steenwinkel, 2001).

2. Studies on mechanism of action: liver and thyroid tumorigenesis in rats

In a 2-year study of toxicity and carcinogenicity in rats, the incidence of hepatocellular adenoma was found to be increased in females given diet containing pyrethrins at a concentration of 3000 mg/kg, while the incidence of thyroid follicular adenoma was increased in males and females given diets containing pyrethrins at a concentration of 1000 or 3000 mg/kg (Goldenthal, 1990). Thus, for mechanistic studies on liver and thyroid tumorigenesis, groups of 15 male and 15 female Sprague-Dawley Crl:CD(SD)IGS BR rats (aged 12–13 weeks) were given diets containing pyrethrins (purity, 57.03%) at a concentration of 0, 100, 3000, or 8000 mg/kg (females) and 0 or 8000 mg/kg (males) for 7 and 14 days. Additional groups were treated for 42 days and sacrificed either directly after completion of treatment or after a 42-day recovery period. Mean intakes of pyrethrins were 0, 6.02–7.68, 163–223 or 262–618 mg/kg bw per day for females, and 0 or 300–503 mg/kg bw per day for males. Further groups of 15 male and 15 female rats were given diets containing phenobarbital (purity, >99%) at a concentration of 1558 mg/kg (equal to 83 mg/kg bw per day) for males or 1498 mg/kg (equal to 85 mg/kg bw per day) for females. However, because of the resulting drowsiness observed in some of the animals, the concentration was reduced to 1200 mg/kg for both sexes on day 8, resulting in mean phenobarbital intakes of 80 mg/kg bw per day for males and 95 mg/kg bw per day for females. All animals were examined for clinical signs, body weights and food consumption assessments. Sampling for analysis of formulated diets was performed during weeks 1, 3 and 6 of the study. Selected animals (eight animals from each group) were given bromodeoxyuridine (BrdU) via a surgically implanted osmotic minipump for 7 days before necropsy. After completion of treatment, animals were killed and blood samples were taken for analysis of clinical chemistry parameters (aspartate amino transferase (AST), alanine transferase (ALT), total protein, total bilirubin), triiodothyronine, thyroxine, reverse triiodothyronine and thyroid stimulating hormone (TSH). A limited necropsy was performed; brain, liver and thyroid glands

Table 1. Results of a study of genotoxicity with pyrethrins in vitro

End-point	Test object	Concentration	Purity (%)	Results	Reference
Gene mutation	L5178Y mouse lymphoma cells; <i>Tk</i> locus	–S9, 0–50 µg/ml	57.03	Negative ± S9 ^{a,b}	Steenwinkel (2001) ^c
		+S9, 0–85 µg/ml (in DMSO)			

S9, exogenous metabolic activation from 9000 × g supernatant fraction of rat liver induced with Aroclor; DMSO, dimethyl sulfoxide

^aTest in duplicate (–S9) or in triplicate (+S9); positive controls included

^bCytotoxicity seen at concentrations of >25 µg/ml; relative total growth was 6% at 50 µg/ml (–S9) and 3% at 85 µg/ml (+S9)

^cStudy complied with GLP (OECD GLP principles 1997) and was performed in accordance with OECD guideline 476 (adopted 21 July 1997)

being weighed and preserved from all animals. Liver and thyroid glands were processed and examined histologically. Examination of slides also allowed a qualitative and quantitative assessment of cell proliferation in the liver and thyroid to be made on the basis of the staining index with BrdU (Finch et al., 2002).

Frozen samples of the liver were sent to another laboratory for analysis of microsomal enzymes; this was performed using a separate protocol. Liver microsomes were prepared and assayed for protein, total cytochrome P450 content and activities of phase I markers of hepatic xenobiotic metabolism (7-ethoxyresorufin *O*-deethylase, 7-pentoxyresorufin *O*-deethylase and testosterone 7 α -, 16 β - and 6 β -hydroxylases) and the phase II enzyme thyroxine UDP glucuronosyltransferase. To allow for any changes in microsomal protein content and liver weight, total cytochrome P450 content and all the enzyme activities measured were expressed as specific content or activity (i.e. per unit of microsomal protein), per gram of liver (i.e. specific activity or content \times microsomal protein content) and by using the absolute and relative liver weight of each animal, i.e. total liver and per liver weight/kg bw, respectively (Lake, 2002).

With the exception of three female rats that were sacrificed prematurely after 7 days of treatment at 8000 mg/kg because of adverse clinical signs, there were no treatment-related clinical effects. In the animals treated with phenobarbital, subdued behaviour and rolling gait were apparent. Body weight and body-weight gain were statistically significantly decreased in males and females at 8000 mg/kg throughout the treatment period; the effect had resolved at the end of the recovery period. A slight but statistically significant decrease in body weight was noted in females at 3000 mg/kg during the first 14 days of treatment, but no significant changes were seen at 100 mg/kg. Animals given phenobarbital showed decreased body weight and/or body-weight gain during the first 7 days of treatment. There was a statistically significant decrease in food consumption in males and females at 8000 mg/kg and in females at 3000 mg/kg over the first 7 days of treatment. In the animals treated with phenobarbital, food consumption was decreased in males for the first 4 days and in females for the first 7 days of treatment, while food consumption was increased at the end of the 14-day period of treatment. Both studies complied with GLP (United States Environmental Protection Agency GLP regulations, 40 CFR Part 160; MHLW, MAFF and METI Japan).

There were slight but statistically significant reductions in AST activity in males at 8000 mg/kg and in females at 3000 and 8000 mg/kg after 42 days of treatment; the decrease was also observed in females at the highest dose after a 42-day recovery period. Activity of ALT was statistically significantly decreased in females at 8000 mg/kg after 7 days and at 3000 and 8000 mg/kg after 42 days of treatment. Total bilirubin concentration was slightly but statistically significantly increased at 8000 mg/kg in males after 14 days and in females after 42 days of treatment. The females treated with phenobarbital showed a statistically significant increase in AST activity after 7 and 14 days and in ALT activity after 14 days, while total bilirubin concentration was slightly increased after 7 days of treatment.

In males receiving diets containing pyrethrins at a concentration of 8000 mg/kg, there were statistically significant reductions in concentrations of thyroxine on days 7, 14 and 42; concentrations of triiodothyronine were reduced on days 7 and 14. In females, concentrations of triiodothyronine were reduced statistically significantly in all treated groups on day 42, while concentrations of reverse triiodothyronine were increased at 3000 and 8000 mg/kg at all time-points, including during the recovery period. Concentrations of thyroid

Table 2. Serum concentrations of thyroxine/triiodothyronine (ng/ml) in rats given diets containing pyrethrins or phenobarbital

Sex	Dietary concentration (mg/kg of feed)	Treatment period			
		7 days	14 days	42 days	42 days +42 days recovery
Males	0	3.69 / 83.12	2.91 / 72.98	3.96 / 75.11	3.21 / 79.92
	8000	2.55***/61.62***	2.24** / 65.23*	3.11** / 68.18	3.63 / 78.07
	Phenobarbital	2.51*** / 71.34**	2.22** / 59.98***	—	—
Females	0	2.36 / 89.24	2.59 / 89.44	2.34 / 98.81	2.48 / 90.05
	100	2.78 / 88.03	2.13* / 83.82	1.89 / 87.44*	2.21 / 89.53
	3000	2.67 / 83.19	2.23 / 86.27	2.82 / 84.09**	2.00 / 85.46
	8000	2.19 / 76.31	2.79 / 84.71	3.00* / 88.90*	2.16 / 85.46
	Phenobarbital	1.64** / 80.48	1.41*** / 84.63	—	—

From Finch et al. (2002)
p* < 0.05, *p* < 0.01, ****p* < 0.001

Table 3. Serum concentrations of thyroid stimulation hormone (ng/ml) in rats given diets containing pyrethrins or phenobarbital

Sex	Dietary concentration (mg/kg of feed)	Treatment period			
		7 days	14 days	42 days	42 days +42 days recovery
Males	0	4.26	4.46	2.68	4.50
	8000	6.28	7.69**	6.95**	2.92
	Phenobarbital	6.11	10.25***	—	—
Females	0	1.87	1.97	2.07	1.86
	100	2.57	2.25	2.14	1.77
	3000	3.79***	4.47***	3.57	1.73
	8000	4.77***	7.88***	7.82***	1.75
	Phenobarbital	4.13***	3.99**	—	—

From Finch et al. (2002)
p* < 0.01, *p* < 0.001

stimulating hormone were statistically significantly increased in males on days 14 and 42, while in females concentrations of thyroid stimulating hormone were statistically significantly increased at 3000 and 8000mg/kg on days 7 and 14 and at 8000mg/kg on day 42. No effects were observed in females at 100mg/kg. For analysis of thyroxine, triiodothyronine and thyroid stimulating hormone, the results for animals treated with phenobarbital were similar for both sexes.

No histopathological effects were seen in animals receiving pyrethrins at a dietary concentration of 100mg/kg. Follicular cell hypertrophy of thyroid glands was found at 3000 or 8000mg/kg in 40–100% of animals at days 14 and 42 or at each time-point, respectively, correlating with increases of approximately 20–50% in organ weight. BrdU-labelling indices in the thyroid in animals killed after 14 days were three- to seven-fold greater than those in controls. Liver cell hypertrophy was found in 60–100% of animals at 3000 or 8000mg/kg at each time-point, correlating with increased liver weights of approximately 30–60% greater than values for controls. BrdU-labelling indices in the liver in the animals killed after 7 or 14 days were three- to five-fold greater than the values for the controls. Results were similar for animals treated with phenobarbital.

Table 4. Weight of the thyroid gland (mg)^a/incidence^b of animals with histological thyroid follicular cell hypertrophy in rats given diets containing pyrethrins or phenobarbital

Sex	Dietary concentration (mg/kg of feed)	Treatment period			
		7 days	14 days	42 days	42 days +42 days recovery
Males	0	21.8 / 0	23.4 / 0	28.2 / 0	30.5 / 0
	8000	25.6 / 7	29.4** / 15	37.4*** / 13	33.9 / 0
	Phenobarbital	26.8* / 9	29.4*** / 15	—	—
Females	0	19.4 / 0	19.8 / 0	22.5 / 0	22.9 / 0
	100	19.0 / 0	19.1 / 0	21.7 / 0	22.7 / 0
	3000	21.4 / 3	24.0** / 11	25.1* / 10	25.0 / 0
	8000	19.9 / 6	29.0*** / 14	29.8*** / 14	27.8** / 0
	Phenobarbital	20.1 / 0	22.6* / 5	—	—

From Finch et al. (2002)

^aUsing body weight as covariate

^bNo. of animals out of 15

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 5. Liver weight (g)^a/incidence^b of animals with histological liver cell hypertrophy in rats given diets containing pyrethrins or phenobarbital

Sex	Dietary concentration (mg/kg)	Treatment period			
		7 days	14 days	42 days	42 days +42 days recovery
Males	0	18.09 / 0	17.66 / 0	21.66 / 0	21.77 / 0
	8000	23.13*** / 14	26.43*** / 9	30.73*** / 11	22.89 / 0
	Phenobarbital	23.21*** / 15	24.60*** / 15	—	—
Females	0	10.94 / 0	11.79 / 0	13.18 / 0	13.09 / 0
	100	10.77 / 0	11.96 / 0	12.35* / 0	13.38 / 0
	3000	13.65*** / 11	15.00*** / 11	16.04*** / 14	13.82 / 0
	8000	14.70*** / 13	18.51*** / 12	18.95*** / 12	14.09 / 0
	Phenobarbital	13.12*** / 14	14.94*** / 15	—	—

From Finch et al. (2002)

^aUsing body weight as covariate

^bNo. of animals out of 15

* $p < 0.05$, *** $p < 0.001$

Treatment with pyrethrins at a dietary concentration of 3000 or 8000 mg/kg significantly increased hepatic microsomal cytochrome P450 content (114–161% of values for the controls) and significantly induced the activities of 7-ethoxyresorufin *O*-deethylase (133–255% of values for the controls), 7-pentoxoresorufin *O*-deethylase (537–3957% of values for the controls), testosterone 16 β -hydroxylase (567–2633% of values for the controls), testosterone 6 β -hydroxylase (155–440% of values for the controls) and of thyroxine UDP glucuronosyltransferase (146–246% of values for the controls) on days 7, 14 or 42. Treatment with phenobarbital for 7 and 14 days significantly increased hepatic microsomal cytochrome P450 content (153–206% of values for the controls) and significantly induced the activities of 7-ethoxyresorufin *O*-deethylase (140–383% of values for the controls), testosterone 7 α -hydroxylase (222–276% of values for the controls), 7-pentoxoresorufin *O*-deethylase (3160–6057% of values for the controls), testosterone 16 β -hydroxylase (2367–3767% of values for the controls), testosterone 6 β -hydroxylase (211–480% of controls) and thyroxine UDP glucuronosyltransferase (147–232% of values for the controls). Overall, the effects of pyrethrins on the investigated hepatic drug-metabolizing enzymes were qualitatively similar to those of phenobarbital. On the basis of intake in mmol/kg per

day, however, phenobarbital was a significantly more potent inducer of CYP2B (i.e. 7-pentoxyresorufin *O*-deethylase and testosterone 16 β -hydroxylase) and CYP3A (i.e. testosterone 6 β -hydroxylase) forms, being 9.1 and 3.4 times more potent than pyrethrins in male and female rats, respectively. In both sexes, the hepatic effects of pyrethrins were reversible on cessation of treatment.

The Meeting concluded that pyrethrins act via an induction of hepatic microsomal cytochrome P450 enzymes and thyroxine UDP glucuronosyltransferase activity that leads to increased clearance of thyroid hormones, as demonstrated by evidence of a correlation between doses of pyrethrins that do and do not increase liver enzyme activities and perturb the concentrations of thyroid hormone. The studies reviewed also demonstrated that the mechanism by which pyrethrins induce formation of tumours in the liver and thyroid is similar to that of other non-genotoxic agents (i.e. phenobarbital) that induce hepatic drug-metabolizing enzymes in the rat. Such agents exhibit a clear threshold for tumour formation and produce tumours by non-genotoxic mechanisms that are most unlikely to occur in humans (Finch et al., 2002; Lake, 2002).

3. Observations in humans: human exposures to consumer products

The Meeting considered a study designed to analyse incidents of exposure to products containing pyrethrins and pyrethroids reported to the American Association of Poison Control Centers (AAPCC) from 1994 to 1999. The original intent of this review was to focus on natural pyrethrins; however, the method by which the AAPCC categorizes data by does not permit pyrethrins and synthetic pyrethroids to be readily distinguished. AAPCC estimated that a population of 260.9 million people was served by the 64 participating centres in 1999, this representing approximately 95% of the population of the USA. The collected data included information about age and sex of the person exposed, reason for exposure (accidental, intentional, etc.), site of exposure, duration of exposure (acute, chronic, etc.), route of exposure (inhalation, ingestion, etc.), patient management and treatment (treated on site, treated and released, admitted for medical or psychiatric care, etc.), symptoms reported, and medical outcome. Medical outcome was a rating of the severity of the effects, made by a specialist in poison information. The ratings included: no effect (no apparent symptoms exist), minor effect, moderate effect, major effect, and death. Given the self-reporting nature of the poison control system, precise quantification of exposure is not possible.

Sales of household insecticides containing pyrethrins and/or pyrethroids from 1995 to 1999 were estimated to be >250 000 000 product units, suggesting that >1 000 000 000 uses of pyrethrin/pyrethroid-containing products occurred during this period, if one assumes that a unit was used on four occasions. The annual number of exposure reports for these products increased from about 11 000 in 1994 to about 16 000 in 1997, when the number of exposures levelled off; the total number of people exposed by a variety of routes to consumer products containing pyrethrins and/or pyrethroids during the review period was 81 838. Adults (aged ≥ 20 years) accounted for the highest proportion of exposures (45%), while children aged <5 years accounted for 37%. One-third of the exposures were through ingestion, while inhalation, dermal and ocular exposures occurred in 27.8%, 26.2% and 10.7% of the cases, respectively. Unintended exposures were most frequently reported (93.1%) and exposures occurred mostly in or around the home (93%); nearly all cases (95%) involved an acute exposure. Of the 49 331 cases for which medical outcome was known, 30.5 % of cases were asymptomatic and 22.4% of cases were considered to be unrelated to the expo-

sure. Where there were symptoms, they were considered to be minor, moderate or major effects for 38.9%, 7.8% or 0.2% of the cases, respectively. There were no deaths reported in the cases included in this analysis. Thus, >90% of the cases with known outcome were either unrelated to exposure, asymptomatic, or featured symptoms of minor severity. Ingestion was the most frequent route of exposure, but was most often associated with minor medical outcome (owing to very low exposure to the product), while inhalation and ocular routes of exposure were more likely to be associated with a more severe medical outcome. Children aged <5 years were most likely to report ocular symptoms, and children aged 5–9 years were most likely to report ocular and dermal symptoms. Adults reported a wider variety of symptoms, including gastrointestinal, dermal, ocular and respiratory effects. Major effects were reported for 114 cases. Detailed records were available for 56 of these cases and they were reviewed in detail. Only 28 cases could be confirmed as “definitely” or “possibly” major outcome, with respiratory and neurological symptoms being reported most frequently (18 and 15 cases, respectively). Of these 28 cases, only seven cases were known to involve pyrethrins, sometimes in combination with other agents. One of these cases reported the outcome of an unsuccessful suicide attempt. Of the remaining six cases, which were major cases associated with exposure to pyrethrins, in only two cases did the patient have a medical history of asthma. One of these reports described a large accidental exposure to a burst can. These data suggest that people with asthma or allergies were not disproportionately represented in the AAPCC reports (PEGUS, 2001).

Comments

In the test for gene mutation evaluated by the present Meeting, pyrethrins did not induce mutations at the *Tk* locus in L5178Y mouse lymphoma cells. The Meeting reaffirmed the conclusion of the 1999 JMPR, which decided that pyrethrins are not genotoxic.

In mechanistic studies of liver and thyroid tumorigenesis, treatment of rats with pyrethrins at a dietary concentration of 3000 or 8000 mg/kg for 7, 14 and 42 days resulted in significant induction of a number of hepatic microsomal cytochrome P450 enzyme activities, thyroxine UDP glucuronosyltransferase activity, decreased triiodothyronine and thyroxine and increased thyroid-stimulating hormone activity. Additionally, increased liver and thyroid weights in association with increased BrdU-labelling indices in the liver and thyroid, and liver cell and thyroid follicular cell hypertrophy were observed. The studies were somewhat limited in that the choice of concentrations used did not thoroughly assess the dose concordance of the mechanistic events with the induction of tumours (e.g. a dietary concentration of 1000 mg/kg, which produced thyroid follicular adenoma in the long-term study of carcinogenicity in rats, was not tested). Nonetheless, the Meeting concluded that pyrethrins induce the formation of liver and thyroid tumours by mechanisms that appear to be similar to those used by other non-genotoxic, mitogenic substances, e.g. phenobarbital, which produce tumours in rodents, and these tumours are not predictive of hazard in humans at relevant exposures. The Meeting thus concluded that the increased tumour incidences caused by pyrethrins are threshold phenomena of negligible toxicological relevance to humans.

Although the data on human exposure (case reports of 81 838 patients exposed by a variety of routes to consumer products containing pyrethrins and/or pyrethroids) did not permit ready distinction between exposure to natural pyrethrins and synthetic pyrethroids, important inferences can be made about the safety of pyrethrins. Of 49 331 cases with known

medical outcomes, >90% of patients had symptoms that were unrelated to exposure, were asymptomatic, or reported symptoms of minor severity. Major effects of exposure were reported in 114 cases, but only in 28 cases (including 7 cases of people exposed to pyrethrins) could these be confirmed as major outcomes after thorough review of the case reports. Among these 28 cases, respiratory and neurological symptoms were reported most frequently (18 and 15 cases, respectively). There was no evidence that having a history of asthma was disproportionately associated with major adverse outcomes after exposure to pyrethrins.

Toxicological evaluation

The Meeting concluded that the ADI of 0–0.04 mg/kg bw established by the 1972 JMPR and reaffirmed by the 1999 JMPR, and the acute RfD of 0.2 mg/kg bw established by the 1999 JMPR are supported by the new data.

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TEBUFENOZIDE (addendum)

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Explanation

Tebufenozide was first evaluated by the 1996 JMPR, which established an acceptable daily intake (ADI) of 0–0.02 mg/kg of feed on the basis of a no-observed-adverse-effect level (NOAEL) for haematotoxicity of 50 mg/kg, (equal to 1.8 mg/kg bw per day) in a 1-year study in dogs, and of 25 mg/kg (equal to 1.6 mg/kg bw per day) in a two-generation study of reproductive toxicity in rats. At the 1999 JMPR, it was recommended that the acute toxicity of tebufenozide be evaluated as soon as possible. The 2001 JMPR evaluated the acute toxicity of tebufenozide on the basis of the available data. The Meeting established an acute reference dose (RfD) of 0.05 mg/kg bw on the basis of a NOAEL of 5 mg/kg bw per day for haematotoxicity in a 2-week study in dogs. The Meeting noted that it might be possible to refine this estimate using the results of a study designed specifically for this purpose. After data from such a study was submitted, the present Meeting reconsidered the acute RfD for tebufenozide.

Evaluation for acute reference dose

The JMPR in 1996 noted that the LD₅₀ of tebufenozide was >5000 mg/kg bw in mice and rats treated orally, and that toxicity after dermal application in mice and rats or inhalation in mice was also low (dermal LD₅₀, >5000 mg/kg bw; inhalation LC₅₀, >4.3 mg/l of air)

In short-term studies of toxicity, the main effect in mice, rats, and dogs given diets containing tebufenozide was haemotoxicity, with signs of regenerative haemolytic anaemia and compensatory responses in haematopoietic tissues. Dogs were the most sensitive species, with effects being seen after 2 weeks, the shortest interval investigated. In rabbits treated by gavage on days 7–19 of gestation, no signs of maternal toxicity, embryo- or fetotoxicity or teratogenicity were observed at a dose of up to 1000 mg/kg bw per day, the highest dose tested. Similar findings were made in studies of developmental toxicity in rats, in which the NOAEL was also 1000 mg/kg bw per day, the highest dose tested. The 1996 JMPR concluded that tebufenozide and its metabolites are not genotoxic.

Acute haemotoxicity in dogs

A study of acute haemotoxicity in dogs was performed in compliance with good laboratory practice (GLP), with the exception that the test diet was not analysed for confirmation of dose or for homogeneity. There are no applicable guidelines for the design of such a study. Groups of four male beagle dogs were given diets containing technical-grade tebufenozide (purity, 99.0%) at a concentration of 1.08 or 4.30 g/kg (1080 or 4300 mg/kg) for 9 h. These concentrations correspond to mean achieved intakes of 21.9 and 89.4 mg/kg bw. Control animals received normal diet. The homogeneity, stability and concentration of the test material were not investigated in this study. All animals were observed twice per day, and body weight was recorded 2 days before the start of the study and on days 8 and 15. Food consumption was recorded on the first day of the study in the two treated groups only. Five days before treatment and on day 1 (before dosing), day 2 (24 h after exposure), day 8 and day 15, blood samples from the jugular vein of fasted animals were taken for haematology and limited blood chemistry (erythrocyte volume fraction, haemoglobin concentration, erythrocyte count, methaemoglobin, Heinz bodies, reticulocytes, platelet count, erythrocyte morphology, erythrocyte indices (mean corpuscular haemoglobin; mean corpuscular volume; mean corpuscular haemoglobin concentration; and total bilirubin). No ophthalmological examinations, necropsy or histopathology were undertaken in this study (for humane reasons; the animals were reassigned after this study).

All the animals survived until the end of the observation period. There were no treatment-related observations during the study. The sporadic occurrence of soft faeces observed in one animal at the highest dose was not considered to be treatment-related, as it had also occurred in this animal before treatment. There was no effect of treatment on body weight; animals in all three groups (except two of the dogs at the highest dose) exhibited minor weight loss over the duration of the study. There were no obvious or statistically significant treatment-related changes in any of the haematological parameters, methaemoglobin formation or serum concentrations of total bilirubin.

The NOAEL was 89.4 mg/kg bw, the highest dose tested, on the basis of clinical observations, haematology, methaemoglobin formation and serum concentration of total bilirubin (Stebbins et al., 2002).

Comments

Tebufenozide has low acute toxicity in rats and mice after oral ($LD_{50} > 5000$ mg/kg bw) or dermal ($LD_{50} > 5000$ mg/kg bw) exposure, and in rats after exposure by inhalation ($LC_{50} > 4.3$ mg/l of air). In short-term studies of toxicity in mice, rats and dogs, the main effect was haematotoxicity, with signs of regenerative haemolytic anaemia and compensatory responses in haematopoietic tissues, accompanied by the formation of methaemoglobin. The dog was the most sensitive species, males showing slightly greater changes in several parameters than females (methaemoglobin, reticulocytes and Heinz bodies).

In the study evaluated by the present Meeting, male beagle dogs received tebufenozide in the diet such that intakes of 21.9 and 89 mg/kg bw were achieved. Animals were permitted 9 h to consume the test meal. The diet was not tested for homogeneity, stability or concentration of the test substance but, given the duration of the treatment period, this was not considered to be a serious limitation of the study. No necropsy or histopathology was undertaken, but the study design was adequate for the evaluation of the acute haematotoxicity of

tebufenozide. Blood samples were taken before, and 2, 8 and 15 days after exposure to tebufenozide. Treatment with tebufenozide had no significant effect on clinical signs or haematological parameters, including reticulocyte numbers or concentrations of serum total bilirubin. The NOAEL was 89.4mg/kgbw, the highest dose tested.

On the basis of studies evaluated previously by the Meeting, it was concluded that tebufenozide and its metabolites are not genotoxic. It was also concluded that tebufenozide is not embryo- or fetotoxic, or teratogenic in rats or rabbits at a dose of up to 1000mg/kgbw per day.

Toxicological evaluation

The Meeting considered that the study in dogs was adequate for the establishment of an acute RfD for tebufenozide. Accordingly, an acute RfD of 0.9mg/kgbw was established, based on a NOAEL of 89.4mg/kgbw (the highest dose tested) and a safety factor of 100.

Reference

Stebbins, K.E., Radtke, B.J. & Baker, P.C. (2002) Tebufenozide: Acute red blood cell evaluation in beagle dogs. Unpublished report of study ID 021104 from Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI, USA. Submitted to WHO by Dow AgroScience LLC, Indianapolis, IN, USA.

TERBUFOS

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Explanation

Terbufos is an organophosphorus compound, classified as a systemic insecticide and nematocide, and was last evaluated by the JMPR in 1989, when an ADI of 0–0.0002 mg/kg bw was established. Terbufos was considered by the present Meeting within the periodic review programme of the Codex Committee on Pesticide Residues.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

In an study of metabolism, 16 male Royal Hart Wistar rats were given a single dose of [¹⁴C]terbufos (purity, uncertain; specific activity of 26.4 µCi/mg labelled at the carbon atom of the thiomethyl portion of the parent molecule (or methylene carbon) in ethanol and

water (1:1) at 0.8 mg/kg bw by gavage. Animals were housed individually in metabolism cages. Urine was collected on dry ice at 6, 12, 24, 48, 72, 96, 120, and 168 h after dosing, and faeces were collected and frozen at 12, 48, 72, 96, 120, and 168 h after dosing. After thawing, urine samples were pooled for each time interval. Groups of three animals were terminated at 6, 12, 24, and 48 h after dosing and the final group of four animals was terminated at 168 h after dosing. Livers, kidneys, gastrointestinal tract, muscle, skin, fat and blood were taken at these times.

Radioactivity was extracted from the urine, faeces and tissues, and metabolites in urine, faeces, liver, kidney and muscle were separated and identified. In addition, the amount of $^{14}\text{CO}_2$ in expired air over a 72 h period was determined in one animal that had received a dose of 0.2 mg/kg bw by gavage. There was no indication of overt toxicity. Elimination was relatively rapid and fairly complete. About 90% of the administered dose was recovered by the end of the study. Over the entire duration of the study, approximately 83% of the administered dose was found in the urine, which was thus the major route of elimination; about 72% of the administered dose was excreted by 24 h and 80% was excreted by 48 h. Over the course of the study, about 3.5% of the radiobel was found in the faeces. The recovery of the administered dose reached a peak of 31.2% in the urine by 24 h, and a peak of about 2% in the faeces by 48 h. Tissue concentrations of radiolabel reached a maximum at between 6 and 12 h after dosing. By 168 h after dosing, the concentration of radiolabel in each body tissue examined was <0.1 mg/kg. The total percentage of the administered dose recovered in tissues by 168 h was greatest in the liver (0.34%), followed by the gastrointestinal tract (0.087%), blood (0.036%), kidney (0.034%), muscle (0.024%), skin (0.017%) and fat (0.003%). No $^{14}\text{CO}_2$ was recovered during the designated interval of 72 h.

After extraction and thin-layer chromatography, metabolites that did not contain phosphorus were found to account for about 96% of the radiolabel present in urine. The predominant species appearing by 6 h after dosing was CL 202474, with lesser amounts of CL 99843, CL 99844 and CL 99875 (see Figure 1 and Table 1). Small amounts of parent compound and other species containing phosphorus accounted for most of the remainder of the radiolabel (2–3%) in the urine. Two peaks of radioactivity in urine were not definitively identified.

In the faeces, at 12 h after dosing, about 95% of the radiolabel comprised species containing phosphorus (mostly metabolites CL 92320 and CL 94221, with lesser amounts of CL 94302 and CL 94301). At subsequent time intervals, in addition to CL 92320, non-phosphorus-containing metabolites CL 202474, CL 99843, and CL 99875 predominated. Very little parent compound was found in the faeces.

The metabolites identified in urine and faeces were also observed in tissue extracts; the types of metabolite found at detectable levels depended on the tissue and time after dosing. In liver, kidney and muscle, the approximate ratio of species not containing phosphorus (four metabolites) to those containing phosphorus (e.g. five phosphorus-containing metabolites and small amounts of parent compound) at 6 h after dosing were 2.6, 11 and 6 in liver, kidney and muscle respectively, and about 9, 23 and 7 for these same tissues at 12 h after dosing. Possible sex differences in the metabolic fate of terbufos were not addressed by this study (North, 1973). No statements of compliance with quality assurance (QA) or good laboratory practice (GLP) were provided. The study was not performed according to a specific guideline. Despite the limitations of the study some useful information can be extracted.

Radiolabelled terbufos (purity, >98%; specific activity, 61.4 mCi/g), labelled with ^{14}C at the methylene carbon position of the parent molecule, was administered in corn oil by gavage to groups of fasted male and female Crl:CD®(SD)BR rats. Groups of five male and five female rats were given single oral doses of 0.1 mg/kg bw (lowest dose) or 0.4 mg/kg bw (highest dose) and additional groups of one male and one female were similarly treated and used for collection of volatiles. In the multiple-dose segment of the study, groups of rats were given non-radiolabelled terbufos (purity, 97.8%) as single oral doses of 0.1 mg/kg bw in corn oil for 14 days, followed by a single dose of radiolabelled material of 0.1 mg/kg bw on day 15. Additional groups of one male and one female were similarly treated with terbufos at a dose of 0.1 and used for collection of volatiles. Urine and faeces for all groups were collected 0–6, 6–12, and 12–24 h after the administration of radiolabelled terbufos and daily thereafter until termination at 168 h. Cage rinses were collected as necessary. Volatiles were collected at intervals of up to 7 days after dosing. Radioactivity was extracted from urine, faeces and tissues (blood, bone, brain, fat, ovaries, testes, heart, liver, kidneys, lungs, muscle, spleen, uterus and residual carcass). Metabolites were characterized and identified only in the urine and faeces and only for the 12–24 h interval after dosing.

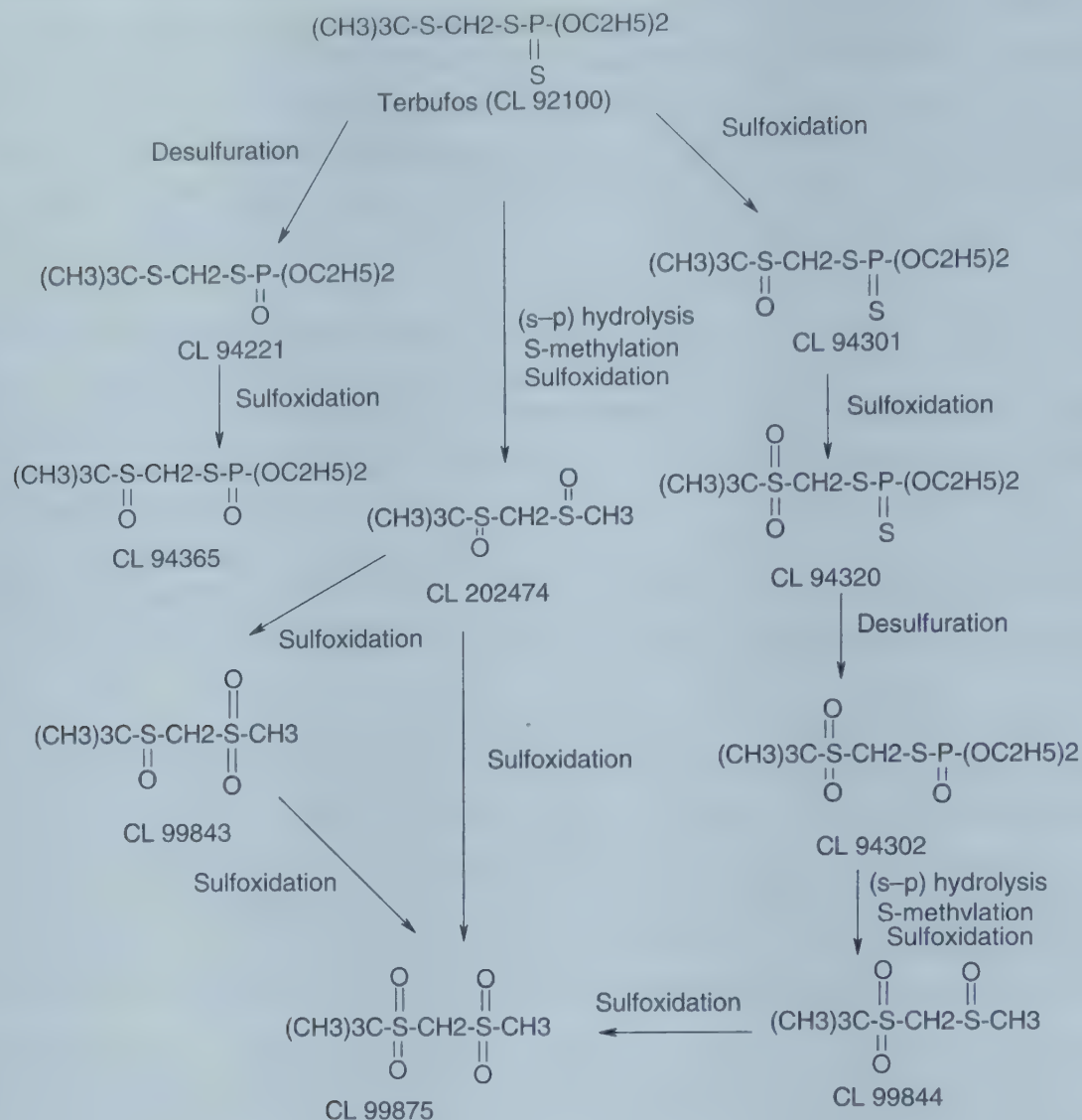
No toxicity was reported. In all the treated groups, total recoveries of the administered radiolabel was about 93–99% in males and 89–96% in females at 168 h after dosing. Relatively rapid elimination, primarily in the urine, indicated fairly fast and appreciable absorption via the gastrointestinal tract. Urinary excretion of radiolabel was 76% and 79% in males and females treated with single low doses of terbufos, and 79% and 69% in males and females treated with single high doses of terbufos, at 168 h after dosing. In animals receiving repeated low doses of terbufos, urinary excretion of radiolabel was 86% and 85% of the administered dose in males and females. Faecal elimination in groups of males and females treated with low and high single doses was 13–17% of the administered dose and in the groups receiving repeated doses was about 5–7% in both sexes. In males and females in all treated groups, much smaller amounts of radiolabel, as a percentage of the administered dose, were found in the tissues (about 0.09–0.15%), in the carcass (about 0.90–2%), in expired CO_2 (about 2–4%) and in volatiles (0.06–0.5%), and most of the radiolabel was eliminated in excreta by 24 h after dosing.

The highest concentrations of residues in tissues at 168 h after dosing were found in animals receiving single, high doses. For all dosing regimens, the highest tissue residues were found in the lungs of both sexes (single low doses, 0.003–0.005 mg/kg; repeated doses, 0.005–0.007 mg/kg; and single high doses, 0.018–0.022 mg/kg) and there was no indication of bioaccumulation.

Metabolites in urine and faeces were characterized only for the period between 12 and 24 h after dosing (Figure 1 and Table 1). Since appreciable amounts of radiolabel were excreted before this interval, the metabolites cannot be reliably quantified relative to percentage of administered dose. In addition, variation in the radiolabel detected in cage rinses for individual animals (about 6–56% of the administered dose) contributed to uncertainty in metabolite quantification during the 12–24 h interval.

For all dosing regimens and for both sexes, about 70–90% of the urinary residue of radiolabel was reported to have been characterized for the 12–24 h period. Of this, about 67–80% of the residue was described as non-phosphorus-containing metabolites, about 1–4% as phosphorus-containing metabolites and about 2–9% as unknown substances (two substances). Of the non-phosphorus-containing metabolites, the major metabolite was CL

Figure 1. Proposed metabolic pathway of terbufos in rats



From Cheng (1992)

Table 1. Terbufos and its metabolites

CL No.	Chemical name	Common name
92,100	<i>O,O</i> -diethyl- <i>S-t</i> -butylthio-methylphosphorodithioate	Terbufos (parent compound)
94,301	Phosphorodithioic acid, <i>S</i> -(<i>t</i> -butylsulfinyl) methyl <i>O,O</i> -diethyl ester	Terbufos sulfoxide
94,320	Phosphorodithioic acid, <i>S</i> -(<i>t</i> -butylsulfonyl) methyl <i>O,O</i> -diethyl ester	Terbufos sulfone
94,221	Phosphorothioic acid, <i>S</i> -(<i>t</i> -butylthio) methyl <i>O,O</i> -diethyl ester	Terbufoxon
94,302	Phosphorothioic acid, <i>S</i> -(<i>t</i> -butylsulfonyl) methyl <i>O,O</i> -diethyl ester	Terbufoxon sulfone
94,365	Phosphorothioic acid, <i>S</i> -(<i>t</i> -butylsulfinyl) methyl <i>O,O</i> -diethyl ester	Terbufoxon sulfoxide
202,474	Methane, (<i>t</i> -butylsulfinyl)(methylsulfinyl)	
99,844	Sulfoxide, (<i>t</i> -butylsulfonyl) methyl methyl	
99,843	Sulfoxide, <i>t</i> -butyl (methyl-sulfonyl) methyl	
99,875	Sulfone, <i>t</i> -butyl (methyl-sulfonyl) methyl	

From North (1973) and Cheng (1992)

202474; lesser amounts of CL 99843, CL 99844 and CL 99875 were found. The only phosphorus-containing metabolite detected was CL 94365; no parent compound was found. During the 12–24 h interval, for all dosing regimens and for both sexes, about 49–80% of the faecal residue of radiolabel was reported to have been characterized. Of this, about 17–34% was described as non-phosphorus-containing metabolites, about 8–44% as phosphorus-containing metabolites, and about 7–22% as unknown substances (five

substances). The non-phosphorus-containing metabolites were CL 202474, CL 99843 and/or CL 99844, and CL 99875. The major phosphorus-containing entity was the parent compound (CL 92100) with lesser amounts of CL 94301 and CL 94365. The proposed metabolic pathway for terbufos in rats, on the basis of the metabolites found in excreta, is depicted in Figure 1. Sulfoxidation and desulfuration of terbufos is followed by hydrolysis of the thiophosphorus bond (S-P), enzymatic S-methylation and then additional S-oxidation.

There were no apparent sex differences in the absorption and metabolic fate of [^{14}C]terbufos in Sprague-Dawley rats on the basis of the results of this study (Cheng, 1992). Statements of compliance with QA and GLP were provided. The protocol was generally consistent with United States Environmental Protection Agency (EPA) Subdivision F Guidelines (November 1982 and revised, 1984).

1.2 Biotransformation

In a study of the biotransformation of terbufos (purity, 91%; apparently purchased from a pesticide factory in China), livers of male Wistar rats (180–220 g) were perfused *in situ* with 100 μl of terbufos (0.1 mol/l, dissolved in methanol and added to the perfusate reservoir) for 1 h at a flow rate of 5 ml/liver per min. Metabolic materials collected from perfusate effluent were separated with a solid-phase extraction cartridge and were characterized and quantified by gas chromatography–infrared spectrometry (GC–IR) and gas chromatography–mass spectrometry (GC–MS). The recovery of terbufos and its metabolites was expressed as a percentage of the concentration of the parent compound entering the liver. Recovery was incomplete, totaling only about 47.13%. Substances in the effluent separated into five major peaks. These were identified as terbufos (40.8%), terbufos oxon (2.13%), and three trialkylphosphorothioate metabolites: metabolite I: $(\text{C}_2\text{H}_5\text{O})_2\text{POSCH}_3$ (hydrolysate of terbufos oxon, 0.13%); metabolite II: $(\text{C}_2\text{H}_5\text{O})_2\text{PSSCH}_3$ (hydrolysate of terbufos, 2.65%); and metabolite III: $(\text{C}_2\text{H}_5\text{O})_2\text{PSSC}_2\text{H}_5$ (methylate of the metabolite II, 1.42%). It was suggested that metabolite III may have formed via a detoxification reaction involving S-adenosyl-L-methionine methyl transferase. The potential for certain trimethyl and triethyl phosphorothioates, such as metabolites I, II and III, to cause cholinergic toxicity and/or pulmonary toxicity in rats by a non-cholinergic mechanism or mechanisms was discussed. Sulfoxide and sulfone metabolites were not detected in the effluent (Li et al., 1999).

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity of terbufos is summarized in Table 2.

Terbufos is of very high acute toxicity when administered by the oral, dermal, or inhalation routes. LD_{50} values for acute oral toxicity in rodents and dogs were similar, ranging from 1.4 to 9.2 mg/kg bw. The acute dermal LD_{50} was about 1 mg/kg bw in rabbits, and the acute inhalation LC_{50} in rats ranged from 0.0012 to 0.0061 mg/l. Clinical signs observed were those typical of cholinergic toxicity and, depending on the study, route and species, included tremors, salivation, exophthalmos, prostration, decreased activity, chromodacryorrhoea, diuresis, piloerection, ataxia, urogenital staining, nasal discharge, anorexia, and laboured breathing. Deaths following acute exposures occurred within minutes to hours or up to a week after administration. With regard to dermal absorption, terbufos is rapidly penetrating after dermal or ocular application.

Table 2. Acute toxicity of terbufos

Species	Strain	Sex	Route	Vehicle	LD ₅₀ (mg/kg bw) or LC ₅₀ (mg/l)	Purity (%)	Reference
Mouse	CF1 albino	Female	Oral	Corn oil	5.0	85.8	American Cyanamid Company A72-95 (1972a)
Mouse	CF1 albino	Female	Oral	Corn oil	9.2	96.7	Morici (1972)
Mouse	CF-1 albino	Male	Oral	Corn oil	3.5	96.7	Morici (1972)
Rat	Wistar (RH albino)	Female	Oral	Corn oil	9.0 ^b	96.7	Morici (1972)
Rat	SD (CrI:CD(SD)BR)	Female	Oral	Corn oil	1.4 ^d	89.7	Bradley (1996) ^a
Rat	SD (CrI:CD(SD)BR)	Male	Oral	Corn oil	3.2 ^d	89.7	Bradley (1996) ^a
Rat	Wistar RH albino)	Male	Oral	Corn oil	1.6 ^d	85.8	American Cyanamid Company A72-95 (1972a)
Rat	Wistar (RH albino)	Male	Oral	Corn oil	4.5 ^b	96.7	Morici (1972)
Rat	SD (CD)	Female	Inhalation; 4 h, whole body	Administered as a vapour	0.0012 (1.2 µg/l)	89.6	Hoffman (1987) ^e
Rat	SD (CD)	Male	Inhalation; 4 h whole body	Administered as a vapour	0.0061 (6.1 µg/l)	89.6	Hoffman (1987) ^e
Rat	Wistar (RH albino)	Male	Inhalation; 7 h	Administered as a vapour	Could not be calculated ^g	96.7	Morici (1972)
Rabbit	New Zealand white	Female	Dermal	Report stated applied as received ^c	0.93 ^b	89.6	Fischer (1985)
Rabbit	New Zealand white	Male	Dermal	Report stated applied as received ^c	0.81 ^b	89.6	Fischer (1985)
Rabbit	Albino	Male	Dermal	Report stated applied as received ^c	1.0	85.8	American Cyanamid Company A72-95 (1972a)
Rabbit	Albino	Male	Dermal	Report stated applied as received ^c	1.1	96.7	Morici (1972)
Dog	Beagle	Female	Oral	Report stated applied as received ^c	6.3 ^d	96.7	Morici (1972)
Dog	Beagle	Male	Oral	Report stated applied as received	4.5 ^d	96.7	Morici (1972)

Although reports for most of these studies (except as footnoted below) were summary in nature and did not contain GLP or QA statements, protocols appeared to be generally consistent with the intent of EPA Subdivision F Guidelines (1982 or 1984, revised)

^aDetailed report contained QA and GLP statements, but stated there was no confirmation of the concentration of test material; the protocol was consistent with US EPA Subdivision F Guidelines (1982 or 1984, revised)

^bReport stated that animals were not fasted

^cTest material was a liquid

^dReport stated that animals were fasted

^eTest material in a gelatin capsule was administered to fasted animals

^fDetailed report contained QA and GLP statements and protocol was consistent with US EPA Subdivision F Guidelines (1982 or 1984, revised)

^gTen animals were exposed for 7 h at 25°C to air that was near-saturated with product vapour at a nominal chamber concentration of 1.99 mg/l. There were two deaths, one on day 5 and the other during days 6–14 after dosing. Clinical findings described as transient irritation and discomfort were present at 0–15 min after dosing and the lung of one survivor was abscessed at necropsy. The findings of this study are inconsistent with those of Hoffman, 1987

(a) *Ocular and dermal irritation*

Rabbit

In a study of primary skin irritation, 0.5 ml of technical-grade terbufos (purity, 96.7%) was applied "as received" to shaved rabbit skin for 24 h under an impervious patch. The product was extremely toxic by the dermal route when administered in a single treatment; all rabbits (number not specified) died within 24 h after dosing. All animals exhibited signs of cholinesterase inhibition before death. The product (a liquid) was said to penetrate rabbit skin and mucous membranes very easily. No indications of dermal irritation or corrosion were reported (Morici, 1972).

In a second study of primary skin irritation, a single application of 0.25 ml of technical-grade terbufos (purity, 85.8%) was administered "as received" to shaved rabbit skin in a similar protocol to that of Morici (1972), with similar results. All animals died within 24 h after dosing and showed signs of cholinesterase inhibition before death. No skin irritation was reported (BASF, 1972a).

In a study of primary eye irritation, 0.1 ml of technical-grade terbufos (purity, 96.7%) was applied "as received" to the conjunctival sacs of six rabbits. The product was extremely toxic by the ocular route when administered in a single treatment; all animals died on the day of dosing and were observed to exhibit signs of cholinesterase inhibition before death. The product (a liquid) was said to penetrate rabbit skin and mucous membranes very easily. No indications of ocular irritation were reported (Morici, 1972).

In a second study of primary eye irritation, a single application of 0.1 ml of technical-grade terbufos (purity, 85.8%) was introduced "as received" into the conjunctival sacs of six rabbits in a protocol similar to that of Morici (1972), with similar results. All animals died 2–3 h after dosing and were observed to exhibit signs of cholinesterase inhibition before death. No ocular irritation was reported (BASF, 1972a). Although the reports for most of these studies were summary in nature and did not contain statements of compliance with GLP or QA, protocols appeared to be generally consistent with the intent of EPA Subdivision F Guidelines (1982 or 1984, revised).

(b) *Dermal sensitization*

A study of skin sensitization of technical grade terbufos was not performed owing to the severe toxicity observed in the studies of primary skin and eye irritation.

2.2 *Short-term studies of toxicity*

(a) *Oral administration*

Mice

In a preliminary study, groups of 10 male and 10 female albino CF1 mice were given diets containing technical-grade terbufos (CL 92 100; purity, 96.7%) at a nominal concentration of 0, 1.0, 4.0 or 16 mg/kg (nominally equivalent to 0, 0.218, 0.911 and 3.30 mg/kg bw per day in males and 0, 0.286, 0.988 and 3.70 mg/kg bw per day in females) for 31 days. Data on analysis of concentrations of test material in the diet were not provided, so test material intake and stability in the feed could not be confirmed. Very few parameters were evaluated. Cholinesterase activity was not assessed. Six females at the highest dose were found dead between days 5 and 14 and one female at the lowest dose was found dead on

day 9. Autolysis prevented attempts to determine the cause of death. Mortality was, however, only notable in females receiving the highest dose (60%), therefore it is possible that the increase in mortality was related to treatment. Decreases in body weight and food consumption were reported in males and females at the highest dose. Weights of the two organs examined (liver and kidney) in four to five animals of each sex per group treated with terbufos were not statistically significantly different from those of respective control groups. For the parameters examined, no effects were observed in other groups. It was reported that upon gross pathological examination of four to five animals of each sex per group at study termination, no gross lesions were found that were attributed to treatment (gross pathology data were not provided). The no-observed-adverse-effect level (NOAEL) in males and females was nominally 4.0 mg/kg (nominally equivalent to 0.911 mg/kg bw per day in males and 0.988 mg/kg bw per day in females; intake of test material could not be confirmed) on the basis of decreases in body weight and food consumption in both sexes and mortality of 60% at the dose above in females only (Morici, 1972). No statements of compliance with QA or GLP were provided and the study was not performed to address a specific guideline.

Rats

In a short-term feeding study, groups of five male and five female Sprague-Dawley rats (aged 4 weeks at study initiation) were given diets containing technical-grade terbufos (CL 92 100; purity, 90.1%), prepared in a vehicle of corn oil and methylene chloride (1:1), at a concentration of active ingredient of 0, 0.125, 0.250, 0.500, 1.00, 3.00 or 6.00 mg/kg per day (equal to 0, 0.020, 0.039, 0.080, 0.16, 0.49, and 0.77 mg/kg bw per day in males and 0.017, 0.033, 0.066, 0.132, 0.409 and 0.750 mg/kg bw per day in females, respectively) for 14 days. The parameters evaluated were limited, including observations for mortality, morbidity and clinical signs of toxicity (data for individual animals were not provided for signs), measurement of body weight and food consumption, organ weight determinations (liver and kidney only) and a gross examination at study termination. Plasma and erythrocyte cholinesterase activity was assessed before treatment and on days, 1, 4, 7, and 14 in the control groups and in groups of males and females receiving the four lowest doses (i.e. 0.020, 0.039, 0.080 and 0.16 mg/kg bw per day). Cholinesterase inhibition was determined relative to the value for the appropriate concurrent control group. Brain cholinesterase activity was not measured, and clinical chemistry, haematological, urine and histopathological examinations were not conducted in this study.

There were two deaths; two females in the group receiving the highest dose (6.00 mg/kg) died or were sacrificed in a moribund condition on days 11 and 13 from treatment-related causes, respectively. Before death, the animals exhibited severe tremors, salivation and prostration. Clinical signs of toxicity in males and females were reported to start on day 2 and last until termination in the group receiving the highest dose (6.00 mg/kg) and included, initially, ataxia, tremors and miosis. From day 7 on, more severe tremors developed and exophthalmos and piloerection were also observed. At 3.00 mg/kg, clinical signs (slight tremors) were also noted in both sexes from day 4 until the end of the study. Signs of toxicity were considered to be treatment-related and were not observed at lower doses. Changes in body weight and/or food consumption seen at the two highest doses were also considered to be related to treatment. Statistically significant decreases in body weight and body-weight gain relative to respective control groups were observed in both sexes (being more severe in females) at 6.00 mg/kg during both weeks. Food consumption was also statistically significantly decreased in males and females at the highest dose in weeks 1 and 2. At 3.00 mg/kg, body weight was reduced (statistically significantly in males) during the first

week only in both sexes, while food consumption did not appear to be affected. Statistically significant decreases in liver and kidney absolute weights and weights relative to body weight were observed in both sexes at 6.00 mg/kg, but terminal body weights were also reduced. At gross necropsy, there were no findings that were attributed to treatment with the test material in any group.

At 1.00 mg/kg (the highest dose assessed), plasma cholinesterase activity was statistically significantly inhibited by 21–37% at all time-points during treatment in males, and by 27–38% on days 4, 7, and 14 in females. At 0.500 mg/kg, plasma cholinesterase was statistically significantly inhibited on day 4 in males (15%) and on days 4 and 7 in females (23%), but no significant inhibition was observed on day 14 in either sex. Erythrocyte cholinesterase was statistically significantly inhibited at 1.00 mg/kg (the highest dose assessed) in males by 51–61% and in females by 40–52%, on days 4, 7 and 14. At 0.500 mg/kg, a statistically significant reduction in erythrocyte cholinesterase activity was noted on these same days, ranging from 22% to 25%, in males. In females at this dose, a significant decrease in erythrocyte cholinesterase activity of only 16% was seen on day 4. This finding in females was not considered to be of toxicological relevance, although the study authors considered that the decreases in erythrocyte cholinesterase activity in both sexes at this dose were associated with administration of the test material. If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, and considering that brain cholinesterase activity was not measured in this study, the NOAEL was 0.250 mg/kg (equal to 0.0039 mg/kg bw per day) in males and 0.5 mg/kg (equal to 0.066 mg/kg bw per day) in females on the basis of statistically significant inhibition of erythrocyte cholinesterase activity (Fischer, 1978).

A statement of compliance with QA, but not with GLP, was provided. The study was preliminary in nature and was not conducted to fulfil a particular guideline.

In a preliminary study performed to aid in selection of doses for a study of subchronic neurotoxicity, groups of five male and five female albino rats (outbred) (CrI:CD®(SD)IGS BR VAF/Plus®) (aged 6 weeks at study initiation) were given diets containing technical-grade terbufos (AC92100; purity, 89.7%) (dissolved in acetone, mixed with a GRIT-O'Cobs® carrier) at a concentration (adjusted for purity) of 0 (acetone and carrier), 1.0, 5.0 or 6.0 mg/kg in males (equal to 0, 0.11, 0.55 and 0.67 mg/kg bw per day, respectively) and of 0 (acetone and carrier), 0.5, 3.0 or 4.0 mg/kg in females (equal to 0, 0.06, 0.33, and 0.43 mg/kg bw per day, respectively) for at least 21 days. Diets were made available to animals until termination on day 22. The number of parameters evaluated was limited and included observations for general condition, mortality and clinical signs of toxicity, and measurement of body weight and food consumption. Plasma, erythrocyte and brain (one-half homogenate) cholinesterase activities were measured from samples obtained and processed on day 22 at study termination and stored frozen at –70°C until analysis. Cholinesterase inhibition was determined relative to the value for the appropriate concurrent control group. Clinical chemistry, haematological, urine, organ weight, and gross and histopathological evaluations were not conducted in this study.

No animals died and no clinical signs of toxicity were observed. Statistically significant decreases in body-weight gain relative to respective control groups, considered to be treatment-related, were noted during each of the 4 weeks in males at the highest dose (6.0 mg/kg) and during weeks 1 and 2 in females at the highest dose (4.0 mg/kg). Food consumption was statistically significantly decreased only in males at the highest dose during

the first week of the study. Treatment-related decreases in blood and brain cholinesterase activities were observed in males and females at the two higher doses. In males at 5.0mg/kg and 6.0mg/kg, plasma cholinesterase activity was statistically significantly inhibited by 73% and 85%, respectively. In females, plasma cholinesterase activity was inhibited at 3.0mg/kg and 4.0mg/kg by 84% (not statistically significant) and 94% (statistically significant), respectively. Erythrocyte cholinesterase activity was statistically significantly inhibited by 98% and 99% in at 5.0mg/kg and 6.0mg/kg, respectively. A smaller inhibition of erythrocyte cholinesterase of about 35% was noted in males at 1.0mg/kg. In the study report, the finding was not considered to be associated with the administration of test material, as it was not statistically significant and there were no reductions in either plasma or brain cholinesterase activity in males at this dose. It may, however, have been related to treatment as the values for cholinesterase activity for four of the five animals in this group fell below those in the control group in replicate assays. In females, at 3.0mg/kg and 4.0mg/kg, respectively, erythrocyte cholinesterase was inhibited by 99% to 100%. Statistically significant decreases in brain cholinesterase of 64% and 81% were observed at 5.0mg/kg and 6.0mg/kg in males, respectively, as were statistically significant decreases in females at 3.0mg/kg and 4.0mg/kg, of 68% and 84%, respectively.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect and brain (not erythrocyte) cholinesterase inhibition and clinical signs of toxicity are considered to be relevant effects for terbufos, the NOAEL was 1.0mg/kg in males (equal to 0.11 mg/kg bw per day) and 3.0mg/kg in females (equal to 0.06 mg/kg bw per day) on the basis of statistically significant inhibition of brain cholinesterase inhibition at the next highest dose (Mandella, 1999). Statements of compliance with QA and GLP were provided. This study was preliminary in nature and was not conducted to fulfil a particular guideline.

In a preliminary feeding study, groups of 10 male and 10 female albino RH Wistar rats were given diets containing technical-grade terbufos (CL 92 100; purity, 96.7%) at a nominal concentration of 0, 0.125, 0.5 or 2.0 mg/kg (nominally equivalent to 0, 0.012, 0.069 and 0.299 mg/kg bw per day in males and 0, 0.012, 0.053, and 0.212 mg/kg bw per day in females) for 31 days. Data on analysis of test material levels in the diet were not provided so test material intake and stability in the feed could not be confirmed. Study parameters examined included observations for appearance, mortality, and measurement of body weight and food consumption. In five animals of each sex per group (when possible), haematological and limited clinical chemistry evaluations (glucose, urea nitrogen and glutamic-pyruvic transaminase), liver and kidney weight measurements and a gross examination were conducted. At the end of the study, blood (from fasted animals) and brain samples (one-half of the cerebrum) were taken from five animals of each sex per group for determination of cholinesterase activity. Brain samples were stored frozen until analysis, and assays were conducted on homogenates. Inhibition of cholinesterase activity was determined relative to the value for the appropriate concurrent control group.

There were many deaths in the study; four males at the highest dose died on day 3, 17, 31 or 31, one male at the intermediate dose died on day 12, and one male at the lowest dose died on day 31. One female at the highest dose was terminated in a moribund condition on day 24, two females at the intermediate dose died on day 9 or 24 and one control female died on day 31. The deaths of five unspecified animals were thought to be the result of a respiratory infection, and autolysis prevented attempts to determine the cause of death in the remainder (again, unspecified). Therefore, it was not possible to ascertain whether any of the deaths were related to treatment. There was no apparent effect of treatment on

body weight, food consumption, on measured haematological and clinical chemistry parameters, or on organ weights in any group treated with terbufos. Cholinesterase activity was statistically significantly inhibited only at the highest dose in both sexes. Statistically significant inhibition of cholinesterase activity in plasma (57%), erythrocytes (36%) and brain (28%) was noted in males at the highest dose, as was (mostly) a statistically significant inhibition in plasma (68%), erythrocytes (37%; not statistically significant), and brain (53%) in females at the highest dose. These decreases were attributed to treatment. There was also a decrease of 29% in erythrocyte cholinesterase activity in males at the intermediate dose; although not statistically significant, this was considered to be a possible result of treatment owing to the magnitude of the decrease. It was reported that upon gross pathological examination of four to five animals of each sex per group at study termination, no gross lesions were found that were attributed to treatment (gross pathology data were not provided). An overall NOAEL could not be identified in this study because insufficient information was provided about the mortality that occurred at all doses in males and at the intermediate and highest dose in females. There were a number of unspecified deaths that were judged likely to be the result of infection in the animal facility. In addition, intake of test material could not be confirmed (Morici, 1972). No statements of compliance with QA or GLP were provided. This study was not performed according to a specific guideline.

In a feeding study, groups of 20 male and 20 female Sprague-Dawley rats were given diets containing technical-grade terbufos (purity, 90.1%; prepared in corn oil and methylene chloride, 1:1) at a concentration (adjusted for purity) of 0 (vehicle), 0.125, 0.250, 0.500 or 1.000 mg/kg (equal to mean intakes of test substance of 0, 0.011, 0.021, 0.041, and 0.082 mg/kg bw per day in males and 0, 0.012, 0.023, 0.048 and 0.095 mg/kg bw per day in females) for 3 months. Parameters evaluated included observations for mortality and clinical signs of toxicity, assessments of body weight, food intake and food efficiency, ophthalmoscopic, haematological, and clinical chemistry evaluations, urine analysis, organ weight determinations, a macroscopic examination in all animals and a microscopic evaluation of organs and tissues in animals at the highest dose and in the control group only. The heart, liver, and kidney, any gross lesions or masses, and any other tissues, as indicated by findings at the highest dose, were examined microscopically in all animals. Plasma and erythrocyte cholinesterase activities were measured on day 1, and at weeks 1 and 2 and months 1, 2 and 3 in 10 animals of each sex per group. Brain cholinesterase activity was measured in 10 animals of each sex per group at study termination. Inhibition of cholinesterase activity was determined relative to the value for the appropriate concurrent control group.

One female at 0.5 mg/kg was mistakenly sexed as male until week 4, when the animal was put with other females in the group. All animals survived until the end of the study, except for one female and one male at 0.5 mg/kg, which died of accidental causes on days 8 and 51. No clinical signs of toxicity were observed and there were no obvious effects of treatment on body weight, food consumption, food efficiency, haematological, clinical chemistry, or urine analysis parameters at any dose. Slight statistically significant increases in liver weight to body weight ratios but not absolute weights in females at the two highest doses were not considered to be of biological relevance. Plasma cholinesterase activity was statistically significantly decreased only at the highest dose, at which activity was inhibited throughout the study in both sexes. At study termination, decreases in activity at the highest dose were 33% in males and 52% in females. Erythrocyte cholinesterase and brain cholinesterase activities were not affected by treatment. The macroscopic examination was not remarkable. Upon microscopic examination of tissues and organs, increases were observed in the incidence of mandibular lymph node hyperplasia at the highest dose in

males (20%) and females (70%) compared with that in male control animals (6.25%) and female control animals (32%). Also, the incidence of mesenteric lymph node hyperplasia was increased in females at the highest dose (50% compared with 25% in the control group), but not in males at the highest dose (25% compared with 30% in the control group). There was no clear association with treatment for these or other histopathology findings in the study. In the study report, the mesenteric lymph node lesions were considered to be related to nematodiasis. Other groups treated with terbufos were not examined for the incidence of either mandibular or mesenteric lymph node hyperplasia. Some respiratory tract lesions that occurred with similar frequency in control groups and in groups treated with terbufos were ascribed to chronic murine pneumonia (said to be a common finding in rodent colonies). If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL was ≥ 1.00 mg/kg (the highest dose tested) in males and females (equal to 0.082 mg/kg bw per day in males and 0.092 mg/kg bw per day in females) (Daly & Knezevich, 1979). A statement of compliance with QA, but no GLP statement, was provided. The protocol was generally consistent with US EPA Subdivision F Guidelines (November 1982 and 1984, revised).

In a study of toxicity, groups of 30 male and 30 female CD® (Sprague-Dawley derived) COBS® rats were given diets containing technical-grade terbufos (AC 92100; purity, 89.6%) (prepared in corn oil and methylene chloride, 1:1) at a concentration of 0 (vehicle only), 0.125, 0.5, or 1.0 mg/kg (equal to 0, 0.007, 0.028, and 0.055 mg/kg bw per day for males and 0, 0.009, 0.036, and 0.071 mg/kg bw per day for females) for 1 year. Parameters assessed included observations for general health and mortality, clinical signs of toxicity, measurement of food consumption and body-weight changes, haematological and clinical chemistry determinations, urine analysis, organ weight measurements and ophthalmoscopic and macro- and microscopic pathology examinations. Plasma and erythrocyte cholinesterase activities were assessed in 10 animals of each sex per group at week 6, months 3 and 6, and at study termination. Brain cholinesterase activity was determined at study termination. Inhibition of cholinesterase activity was calculated relative to values for concurrent controls.

There were six deaths in the study; two control animals (one male on day 306 and one female on day 271) and two animals at the intermediate dose (one male on day 351 and one female on day 262) were terminated in a moribund condition. One male at the intermediate dose was found dead on day 310 and one female at the highest dose died accidentally on day 97. There was no obvious pattern in the deaths in the animals treated with terbufos that would suggest a relationship with administration of the test material. There was no clear relationship with treatment for the slight increases observed mostly during the last half of the study in the group of females at the highest dose in the incidence of excess lacrimation, chromodacryorrhoea and alopecia compared with the control and other treated groups. As similar increases were noted at lower doses in males, the findings in females were considered to be likely to be caused by random variation. There were no clear effects of treatment on body weight. Variation was noted among groups in food consumption over the course of the study, but there was no consistent pattern of findings that would clearly indicate an effect of treatment in groups of males or females treated with terbufos. There was no evidence that the results of the urine analyses or those of the ophthalmoscopic and haematological examinations were related to treatment. Statistically significant decreases, of small magnitude, in blood urea nitrogen and bilirubin at termination in females at the highest dose were not considered to be clearly of adverse significance in the absence of other findings. Clinical chemistry evaluations were otherwise not remarkable. Slight, statistically signifi-

cant reductions were observed at the highest dose in the absolute weight and weight relative to brain weight (but not relative to body weight) of male testes, and slight statistically significant decreases in the absolute weight and weight relative to brain weight (but not relative to body weight) of the kidney were noted in females at the intermediate and highest doses. These organ-weight changes were not clearly adverse in the absence of other supporting indications of toxicity. No obvious effect of treatment was indicated by the results of the gross and microscopic examinations. There was no evidence of carcinogenicity.

Decreases in cholinesterase activity that could be clearly related to treatment were observed only at the highest dose in both sexes. Plasma cholinesterase activity was statistically significantly inhibited in males at the highest dose by 25% and 29%, respectively, at 6 and 12 months. Statistically significant inhibition of plasma cholinesterase activity (33–51%) was noted at all time-points in females at the highest dose. Erythrocyte cholinesterase activity was not statistically significantly inhibited at any dose or time-point in either sex. Brain cholinesterase activity in males was decreased relative to control values at the lowest, intermediate and highest dose, respectively by 4% (statistically significant), 3% (not statistically significant) and 8% (statistically significant). On the basis of the magnitude and pattern of the response, inhibition at the lowest dose was not considered to be related to treatment. In females, brain cholinesterase activity was statistically significantly inhibited only at the highest dose. Virtually no changes relative to the control group was observed at the lowest and intermediate doses. Although the decrease observed at the highest dose in males and females may have been related to treatment, because of the relatively low magnitude of the response and the absence of clinical signs at this dose, it was not considered to be toxicologically relevant.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect and inhibition of brain cholinesterase activity of 8–10% is not considered to be toxicologically relevant, the NOAEL was 1.0 mg/kg (equal to 0.055 mg/kg bw per day in males and 0.071 mg/kg bw per day in females), the highest dose tested (Daly, 1987). This study complied with QA and GLP and was consistent with US EPA Subdivision F Guidelines.

Dogs

In a short-term study to assess cholinesterase activity, groups of four male and four female beagle dogs (except at the highest dose, where two dogs of each sex were used) were given technical-grade terbufos (AC 92 100; purity, 89.6%) at a dose (adjusted for purity) of 0 (vehicle), 1.25, 2.5, 5.0, or 15.0 µg/kg bw per day, administered orally in corn oil in gelatin capsules, once daily in the morning for 29 days. Originally planned for 28 days, dosing was extended by 1 day through a protocol amendment. The number of parameters evaluated was limited and included observations for mortality, morbidity and clinical signs of toxicity, and measurement of body weight and food consumption. Plasma and erythrocyte cholinesterase activities were assessed before dosing and after 1, 2, and 4 weeks of treatment from blood samples collected before dosing on the given day. Brain cholinesterase activity was determined at study termination in samples from the cerebrum and cerebellum obtained 20–24 h after administration of the last dose. In the study report, cholinesterase inhibition in the plasma and erythrocytes was determined and statistically analysed relative to values obtained before the start of dosing. Inhibition of cholinesterase activity in the brain was determined relative to the value for the appropriate concurrent control group. Clinical chemistry, haematological, urine, organ weight and gross and microscopic histopathological evaluations were not performed in this study.

There were no deaths in the study and no clinical findings were observed that could be ascribed to treatment in any group. One instance of vomiting was observed in each of two dogs at the lowest dose only. There were no appreciable differences in body weights or food consumption among groups of either sex during the study. Statistically significant decreases in plasma cholinesterase activity ranging from 33% to 37% were noted at the highest dose in both sexes at all time-points and were ascribed to treatment. Inhibition of plasma cholinesterase activity of 20–21% was observed at 5.0 µg/kg bw per day in males after 2 and 4 weeks of treatment and in females at all time-points; these values were not statistically significantly from those for the respective groups of pre-treatment controls and were considered to be of marginal biological relevance in the study report. If inhibition of plasma cholinesterase activity was determined relative to values for the appropriate concurrent controls instead of pre-treatment values (as in the study report), there was not much difference in the magnitude of the decreases calculated by either procedure for either sex at any week or dose, except in females at 2.5 and 5.0 µg/kg bw per day. In these groups, slightly greater decreases in plasma cholinesterase activity were measured relative to values for concurrent controls at all time-points (28–30% at 5.0 µg/kg bw per day, and 18–23% at 2.5 µg/kg bw per day) than relative to pre-treatment values (20–21% at 5.0 µg/kg bw per day, and 10–18% at 2.5 µg/kg bw per day). Under the conditions of the study, there was no effect of treatment in either sex or at any dose on erythrocyte cholinesterase activity (relative to pre-treatment values or to values for the appropriate concurrent controls) or on brain cholinesterase activity relative to the values for the appropriate concurrent controls. If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL was 15 µg/kg bw per day (the highest dose tested) in males and females (Shellenberger, 1987). Statements of compliance with QA and GLP were provided. This was a special study that was not conducted to comply with a particular guideline.

In a preliminary feeding study, groups of two male and two female beagle dogs (aged 8–12 months) were given diets containing technical-grade terbufos (CL 92100; purity, 96.7%) (prepared in a vehicle of corn oil) at a nominal dose of 0, 0.01, 0.05, or 0.25 mg/kg bw per day administered daily for 30 consecutive days. Food consumption was estimated by visual inspection and many animals did not always consume all the food offered. In addition, data on the analysis of concentrations of test material in the diet were not provided, so intake of test material could not be determined with any degree of confidence, and stability of the test material in the feed could not be confirmed. Parameters assessed included observations for appearance, mortality and measurement of body weight and food consumption, haematological and limited clinical chemistry evaluations (plasma glucose, glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase, and urea nitrogen), liver and kidney organ weight measurements and gross examination of all animals. Plasma and erythrocyte cholinesterase activities were assessed before the start of dosing, at week 2 and at study termination in fasted animals. Brain cholinesterase activity was measured at study termination in homogenates of samples taken from the cerebrum. Cerebrum tissue was stored frozen until analysis. Inhibition of cholinesterase activity was determined relative to the values for the appropriate control group.

There were no deaths in the study and no clinical signs of toxicity were observed. On the basis of the estimated food refusal (%) during the study, received doses were as much as 20–35% lower than nominal doses in most animals at the lowest and intermediate doses, and by as much as 35–50% at the highest dose. Over the course of the study, males at the highest dose failed to gain weight and females at the highest dose and one female at the intermediate dose lost weight; these findings were statistically significant for the groups

receiving the highest dose. There was no apparent effect of treatment on organ weights, or on the haematological and clinical chemistry parameters measured. No treatment-related findings were observed during the gross examination. As values for cholinesterase activity were similar in males and females at the same dose, data were pooled for statistical analysis. By week 4, statistically significant decreases in plasma cholinesterase activity of 68% and 84%, and in erythrocyte cholinesterase activity of 35% and 80% were observed at the lowest and intermediate doses, respectively. Brain (cerebrum) cholinesterase activity was statistically significantly inhibited only at the highest dose (by 66%). An overall NOAEL could not be identified because intake of test material could not be reliably estimated (Morici, 1972). No statements of compliance with QA or GLP were provided. This study was not performed to comply with a specific guideline.

In a feeding study, groups of four male and four female beagle dogs (aged 10–14 months, i.e. somewhat older than the age recommended in the guidelines) were given terbufos (AC 92,100; purity not specified) at a nominal concentration of 0 (vehicle only), 2.5, 10.0, or 40.0 mg/l (nominally equivalent to 0, 2.5, 10, and 40 µg/kg bw per day) in corn oil, administered daily for 6 days per week, for 6 months. One ml of the test material in the vehicle was injected via a syringe on top of each dog's daily food ration (kibbled dog chow). Doses were adjusted weekly for each individual animal's body weight. A set amount of treated food (apparently totalling 3300 g per week) was offered for 1 h each day and any food not consumed in that time was removed and weighed in order to measure food consumption and estimate intake of test material. Neither dosing solutions nor treated diets were analysed to confirm content or stability of the test material. Parameters examined included mortality and clinical signs of toxicity, assessments of body weight and food intake, ophthalmoscopic, haematological, and limited clinical chemistry (alkaline phosphatase, blood urea nitrogen, fasting blood sugar, and serum glutamic-pyruvic transaminase) evaluations, urine analysis, weights of selected organs (adrenals, gonads, kidneys, heart and liver), and macroscopic and microscopic examinations. Plasma and erythrocyte cholinesterase activities were measured before the start of dosing and at weeks 0, 4, 12 and 26. Brain cholinesterase activity was assessed at week 26. Although data on cholinesterase activity were provided for individual animals, in the study report data from both sexes were combined for statistical analysis (*t*-test) for each dose and type of cholinesterase activity, as differences between the sexes were not considered to be remarkable. Data on cholinesterase activity from groups treated with terbufos were compared with data for the combined concurrent control group.

Test material intake could not be reliably determined. In addition to a lack of analytical information on the purity, dietary content and stability of the test substance, some animals did not always consume all their food. Since the solution containing the test material was placed on top of the daily food ration, it is not clear how much of the test material was ingested by animals that did not consume the daily food allotment. Female dogs had a greater tendency not to consume the entire meal. The approximate percentage of unfinished meals over the course of the study for all females at each dose was 36% in the control group, 38% at the lowest dose, 40% at the intermediate dose and 23% at the highest dose. Over the duration of the study, some females received only about 80–90% of the intended dose of terbufos. The situation was less severe in males; the approximate percentage of all unfinished meals over the duration of the study for all males at each dose was 31% for the control group, 5% at the lowest dose, <1% at the intermediate dose and 2% at the highest dose. Also, initial body weights varied more than might be desirable (20% difference between some animals of the same sex).

One male in the control group was terminated in a moribund condition and was found to have a colon obstruction and peritonitis. Clinical signs of toxicity were not observed in any animals (individual animal or summary data was not included in the report).

With regard to general condition, one male at the lowest dose, one male at the highest dose, three females in the control group, one female at the highest dose, and possibly one female at the lowest dose and one female at the intermediate dose, had histopathological indications of bronchopneumonia. Body-weight loss (measured over the duration of the study) was noted at termination at week 26 in individual animals in all groups except females at the highest dose. The incidence of weight loss (and average body-weight change) during the study was: in males, 2 (−0.55 kg), 3 (−0.45 kg), 3 (−1.8 kg), and 3 (−0.4 kg), respectively; and in females, 1 (0.35 kg), 1 (0.075 kg), 1 (0.15 kg) and 0 (0.425 kg), in the control group and at the lowest, intermediate and highest dose, respectively. There was no obvious dose-related pattern in these findings. The greater weight loss in the group of males at the intermediate dose was due to one animal that was the heaviest animal in the group at initiation of treatment and that lost 5.7 kg in weight during the study, apparently exhibiting no clinical signs or indications of toxicity, and no unusual findings with regard to food consumption or other parameters examined (except for a slight elevation in erythrocyte sedimentation rate at week 26). The only gross or histopathological finding in this animal was diffuse mild leukocytosis in the liver, which the study report stated could, along with the elevated erythrocyte sedimentation rate, have been related to an infection in this dog near the end of the study. In other animals, there was no clear effect of treatment on the haematological, biochemical, urinary parameters or organ weights assessed or in the gross or histopathological examinations.

Inconsistencies and variability were noted in the data on cholinesterase activity. In the study report, differences between sexes for a given type of cholinesterase activity and at a particular dose were considered to be minimal and data were combined for both sexes for statistical analysis. Under these conditions, there were no statistically significant differences in brain or erythrocyte cholinesterase activity at the end of the study (week 26). Plasma cholinesterase activity at week 26 was statistically significantly decreased relative to values for the combined control group, by 26% at the intermediate dose and 31% at the highest dose; the study report considered these decreases to be minimal but treatment-related. The value for plasma cholinesterase activity from the control male that was terminated in a moribund condition was not included in the calculation, although it was not an outlying value compared with those for the rest of the animals in the group. Data for individual animals were provided. When plasma cholinesterase activity was calculated for each sex separately at week 26, without statistical evaluation, there was no clear effect on plasma cholinesterase activity in males relative to that of the control group, either with the inclusion of the terminated control male (no decrease at the lowest dose, decrease of 35% at the intermediate dose, decrease of 15% at the highest dose) or without it (decrease of 9% at the lowest dose, decrease of 47% at the intermediate dose, decrease of 31% at the highest dose) owing to the lack of a clear dose–response relationship at the intermediate and highest doses. In females at week 26, decreases in plasma cholinesterase activity relative to that of the control group were noted at the intermediate dose (19%) and highest dose (32%), indicating a possible, small effect of treatment, particularly at the highest dose. Erythrocyte cholinesterase activity at the highest and intermediate doses was decreased by 26% and 6%, respectively, when data for males were taken separately (as compared with reduction of 17% and 13% (neither statistically significant) at the highest and intermediate doses, respectively, when

data from both sexes were combined), suggesting a possible minimal, but certainly not clear, effect of treatment at the highest dose.

There were no clear adverse findings associated with treatment in this study, but because nominal concentrations of test material in the diet could not be confirmed, intake of test material could not be reliably estimated and underconsumption of diets containing terbufos was noted, especially in females, a NOAEL could not be identified (Morgareidge, 1973). Statements of compliance with QA and GLP were not provided. The protocol and study conduct were considered to be inadequate according to current standards.

Subsequent to the 6-month feeding study in dogs (Morgareidge, 1973), a study was conducted to attempt to address questions about the 6-days-per-week dosing regimen used in that study, and to determine whether a 7-days-per-week regimen would influence cholinesterase activity, particularly in erythrocytes. Groups of two male and two female beagle dogs (aged 9.5–12 months) were given diets containing technical-grade terbufos (purity, 88%) at a dose of 50 µg/kg bw daily, for (1) 7 days per week for 28 days; or (2) 6 days per week (basal food given on day 7) for 28 days, followed in both groups by a 28 day recovery period without treatment. Five out of the eight dogs in the study had previously been exposed to dichlorvos, another cholinesterase-inhibiting chemical, 4–7 months before their arrival in the testing facility. No other details were provided. A concurrent control group was not included in the study. The test material was prepared as a solution in corn oil, added to a fixed amount of food via a syringe at the rate of 1 ml/kg bw, and given to each dog individually in the morning. If treated food was not eaten within 1 h, it was removed and weighed. It was stated that any food treated with terbufos that was not consumed in the morning was re-administered to the animal in the afternoon (regular feed of plain dog chow), such that any remaining test substance was eaten. Erythrocyte and plasma cholinesterase activities were measured before treatment on days -7 and -6, on days 1, 3, 7, 10, 14, 18, 21 and 28, and after treatment on days 29, 31, 35, 38, 42, 49 and 56. Average cholinesterase inhibition for each group (both sexes combined) was calculated relative to the respective average value before treatment (sexes combined) at each time-point, but apparently these comparisons were not analysed statistically. Statistical analysis was performed for each time-point (both sexes combined) to determine whether there was a significant difference between the 6-day treatment regimen and the 7-day treatment regimen. Animals were observed for general condition, signs of toxicity and body-weight changes during treatment. After the first treatment phase of the study was concluded, a 28-day cross-over phase was performed in which the group of animals dosed for 7 days per week were dosed for 6 days per week for 28 days and the group of animals dosed for 6 days per week were dosed for 7 days per week for 28 days. Erythrocyte and plasma cholinesterase activities were assessed before treatment and on days 1, 3, 7, 10, 14, 18, 21, 25, and 28. There was no recovery period. Brain cholinesterase activity was not assessed in either the first phase or the cross-over phase.

Dosing solutions were analysed four times during the first phase of the study and found to contain 87.6–98.8% of the target amounts (average, about 92%). All animals survived both study phases and no clinic signs of toxicity were observed. In the first phase, initial body weights in some dogs varied considerably; a difference of about 40–50% was noted between some animals in the 7- or 6-day dosing group. Three out of four dogs in both the 7- and 6-day feeding groups lost weight during at least part of the first phase of treatment; this may have been related to treatment, but there were no control groups available

for comparison. Some weight gain was noted in most animals in both groups during the recovery period. Recorded weekly food intakes for individual animals in the first phase were fairly variable week-by-week and between individuals in dogs fed the test material for 7 days per week; it is thus not clear whether all animals in that group ate all the treated food offered. Far less variability was observed in dogs fed test material for 6 days per week, as apparent weekly maximums of 2400 g of food per dog were commonly consumed.

Owing to the small sample size and variability in the data, it was difficult to determine whether there was a sex difference in cholinesterase activity measurements. In the study report, it was assumed that there were no sex differences and group means from data from both sexes combined were compared. In the first phase of the study, on day 28, although data were apparently not analysed statistically, erythrocyte and plasma cholinesterase activities decreased by 14% and 68%, respectively, in the group fed test material for 7 days per week, and by 4% and 40%, respectively, in the group fed test-material for 6 days per week relative to values before treatment (there was no concurrent control group). The difference between the groups treated for 7 days per week and for 6 days per week was not statistically significant with regard to erythrocyte cholinesterase activity at any time-point, either during or after treatment. Differences in plasma cholinesterase activity between the groups treated for 7 days per week and for 6 days per week were statistically significant at weeks 7, 21 and 28. Based on the data provided, recovery to pre-treatment or almost pre-treatment levels was noted during the recovery phase with both dosing regimens, but progress appeared to be a little more rapid on the 6-day regimen and plasma cholinesterase activity seemed to recover more rapidly than erythrocyte cholinesterase activity on either regimen.

In the cross-over phase of the study, after 28 days of treatment, erythrocyte and plasma cholinesterase activities decreased by 18% and 55%, respectively, in the cross-over group fed test material for 7 days per week and by 28% and 52%, respectively, in the cross-over group fed test material 6 days per week, relative to values before treatment. No statistically significant differences were found between the groups on the 6- and 7-day feeding regimens at any time-point assessed. Body-weight losses and variable food intakes were noted with both groups. The study authors concluded that the decrease in cholinesterase activity noted was reversible after cessation of treatment. They also concluded that there were no cumulative adverse effects in erythrocyte or plasma cholinesterase activity on either regimen. In the first phase, however, there did appear to be a slightly larger effect on cholinesterase activity on the 7-day regimen compared with the 6-day regimen, and in the second phase a stronger effect on erythrocyte activity was observed relative to the first phase, possibly implying some carry-over influence between treatments. Owing to the small sample size, the limited protocol (no assessment of brain cholinesterase activity), the lack of a concurrent control group, the use of only one dose, questions about the intake of test material based on food consumption data, the variability in data for individual animals and other uncertainties (e.g. previous treatment of test animals with dichlorvos), this study is not suitable for the identification of a NOAEL or for performing regulatory toxicology assessments for terbufos. No statements of compliance with QA or GLP were provided and the study was not performed to comply with a particular guideline (Berger, 1977).

In a 1-year study, groups of male and female beagle dogs were fed gelatin capsules containing technical-grade terbufos (AC 92,100; purity, 89.6%) at an initial dose (not adjusted for purity) of 0 (vehicle only), 15, 60, 240 or 480 µg/kg bw per day in corn oil for 1 year. Owing to toxicity that resulted in mortality, the dose of 480 µg/kg bw per day was

reduced to 120 µg/kg bw per day 1 day before the start of week 6, and the dose of 240 µg/kg bw per day was decreased to 90 µg/kg bw per day 2 days after the beginning of week 8. Eight animals of each sex were assigned to the vehicle control group and six animals of each sex were assigned to each of the groups receiving terbufos. During weeks 3 and 4, an error occurred and the doses administered were only 5.2% of those intended. Administration of the test material continued until 20–24 h before termination, and animals were terminated during the 5 days following the 1-year period of treatment. Parameters assessed included general condition, mortality, morbidity and clinical signs of toxicity, measurement of body weight and food consumption, and clinical chemistry, haematology, urine analysis, ophthalmoscopy, organ weight, and gross and microscopic evaluations in all animals. Plasma and erythrocyte cholinesterase activity in fasted animals was assessed before treatment, at months 3 and 6 and at study termination. Brain cholinesterase activity in samples from the cerebrum and cerebellum was measured from tissues taken at study termination and stored frozen until analysis. Inhibition of cholinesterase activity was determined relative to the value for the appropriate concurrent control group.

There were three deaths attributable to treatment-related causes. One male and one female at the highest dose died during week 6, while the dose of 480 µg/kg bw per day was being administered. Clinical signs observed in these animals included vomiting, slight tremors and inactivity in the male and tremors, diarrhoea, weak hind legs and excessive salivation in the female. Decreased body weight and food consumption were noted in both dogs. One female at 240/90 µg/kg bw died during week 7, also from causes related to treatment, while receiving the dose of 240 µg/kg bw per day. Clinical findings in this animal included tremors that increased in severity, inactive behaviour, red-tinged faeces, excessive salivation, dehydration, listless behaviour, rough hair coat and decreased body weight and food consumption. One other female at the highest dose was sacrificed in a moribund condition at the start of week 31, owing to causes unrelated to treatment (prolapsed vagina).

Clinical signs of toxicity noted in surviving males at 480 µg/kg bw per day included tremors (slight to more severe) and inactivity. In males at 240 µg/kg bw per day, clinical signs of toxicity included excessive salivation, dehydration, red-tinged faeces, listlessness and inactivity. Symptoms in females were generally more severe than in males at these doses and included tremors (slight to more severe), inactivity, excess salivation, red-tinged faeces, vomiting and weak hind legs at the highest dose (480 µg/kg bw per day), and slight tremors, inactive behaviour, excessive salivation, weak hind legs, diarrhoea, and red-tinged faeces at the lowest dose (240 µg/kg bw per day). Decreases in body weight and food consumption were noted in males at both 480 µg/kg bw per day and 240 µg/kg bw per day and to a greater extent in females at these doses during the early weeks of the study before and just after the decrease in dose. During this time, body-weight decreases reached statistical significance in females at the highest dose, and statistically significant decreases in food consumption were noted for males and females at the highest dose and for females at 240 µg/kg bw per day. Shortly after the two higher doses were lowered to 120 and 90 µg/kg bw per day, respectively, and for the remainder of the study, there was no apparent effect of treatment with terbufos on body weights or food consumption at any dose.

After the two higher doses were reduced, the only clinical findings noted during the remainder of the study, for which an association with treatment could not be dismissed, were two instances of severe convulsions in one female at 120 µg/kg bw per day during weeks 46 and 47. There were no obvious effects of treatment on the ophthalmoscopic, clinical chemistry or urinary parameters assessed or on organ weights after doses of terbufos

of $\leq 120 \mu\text{g/kg}$ bw per day. In males, at month 3, slight statistically significant decreases were found in erythrocyte counts at 90 and $120 \mu\text{g/kg}$ bw per day, and in haemoglobin, erythrocyte volume fraction, and mean corpuscular haemoglobin concentration at $120 \mu\text{g/kg}$ bw per day. Numbers of platelets were also slightly increased (statistically significantly) in males at 90 and $120 \mu\text{g/kg}$ bw per day. Slight, statistically significant decreases in haemoglobin and erythrocyte volume fraction were noted at month 3 in females at $120 \mu\text{g/kg}$ bw per day. These perturbations were transient and had resolved by the next assessment at month 6. They may have been related to treatment at these doses or may have been residual effects from previous dosing at 240 and $480 \mu\text{g/kg}$ bw per day.

Data on cholinesterase activity reported in this study were difficult to interpret owing to variability in the values for individual animals and because of generally inconsistent or sometimes shallow dose–response relationships. Data were apparently not analysed statistically. Plasma cholinesterase activity was inhibited by about 40% or more in all groups receiving terbufos at all time-points in both sexes. At study termination (week 52), plasma cholinesterase activity was inhibited at the lowest, low intermediate, high intermediate and highest doses by 44%, 66%, 67% (shallow dose–response relationship) and 68%, respectively, in males, and by 45%, 68%, 67% (shallow dose–response relationship) and 74%, respectively, in females. Owing to the magnitude of the relative decreases, a relationship to treatment could not be excluded at any dose. At study termination (week 52), erythrocyte cholinesterase activity was lower than the control value at the lowest, lower intermediate, higher intermediate and highest doses in males by 4%, 13%, 18% and 19%, respectively. Similar patterns were found at earlier time-points. The decreases at week 52 were considered to be marginal and the changes of slightly higher magnitude at the two higher doses were considered to be of no toxicological significance. In females, inhibition of erythrocyte cholinesterase activity varied little from control values at the lowest and lower intermediate doses, and ranged from 18% to 28% at the higher intermediate dose and 27% to 35% at the highest dose, at all time-points. At study termination (week 52), erythrocyte cholinesterase activity was lower than the control value in the lowest, lower intermediate, higher intermediate and highest doses in females by 6%, 15%, 20% and 27%, respectively. The magnitude and consistency over time of the change at the highest dose was such that a relationship to treatment could not be dismissed. At week 52, cerebellum cholinesterase activity was not inhibited at any dose (values were 98% to 125% of those of the controls) in males. Cerebrum cholinesterase activity in males was lower than the control value at the lowest, low intermediate, higher intermediate and highest dose by 5%, 10%, 22%, and 22%, respectively. Despite the lack of a dose–response relationship at the two higher doses and the large variability in the data, the magnitude of the response in the brain at the two higher doses was such that a relationship to treatment could not be dismissed. For the same doses, inhibition of cholinesterase activity in the cerebellum in females was 3%, 3%, 12% and 21%, respectively. The magnitude of inhibition in the brain at the two higher doses could not be ignored as a possible effect of treatment; however, the decrease of 12% was not considered to be toxicologically relevant. At the lowest and highest doses, cholinesterase activity in the cerebrum of females was similar to that in controls (although activity fell below the mean for controls by 18% and 24% at the two intermediate doses). Owing to the lack of a dose–response relationship, a treatment-related effect was not readily supported.

Gross lesions seen in the intestinal tracts of animals that died during weeks 6 and 7 had non-neoplastic microscopic correlates. On histopathological examination, the male at $480 \mu\text{g/kg}$ bw per day that was found dead at week 6 had diffuse congestion of the

duodenum, jejunum, ileum and colon, congestion of the lungs, kidneys and liver and fibrous thrombi in the pulmonary vessels of the lungs and in the arteries of the pancreatic mesentery. Microscopic findings in the female at the highest dose found dead at week 6 were marked haemorrhage and congestion in the mucosa and muscularis of the jejunum and areas of necrosis in the muscularis, mucosa and Peyer patches of the ileum (possibly secondary to intussusception). The female at 240 µg/kg bw per day found dead at week 7 had congestion of the muscularis and mucosa of the jejunum on microscopic examination. The study authors considered that these findings were likely to be related to treatment. Other gross and microscopic findings in the study were considered to be incidental and not related to treatment.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, and inhibition of brain (not erythrocyte) cholinesterase activity is considered to be a relevant effect for terbufos, the NOAEL was 60 µg/kg bw per day in males on the basis of a decrease (22%) in cerebral cholinesterase activity for which a relationship to treatment could not be excluded, and 90 µg/kg bw per day in females on the basis of a decrease in cerebellar cholinesterase activity of 21% and instances of severe convulsions in one female for which an association with treatment could not be dismissed (Shellenberger & Billups, 1986). Statements of compliance with QA and GLP were provided and the protocol was consistent with US EPA Subdivision F Guidelines (November 1982 and 1984, revised).

Sheep

In a feeding study, groups of three wethers (males) were given diets containing technical-grade terbufos (purity, 89.8%; in corn oil, diluted with an equal volume of methylene chloride) at a concentration (not adjusted for purity) of 0 (with vehicle only), 0.01, 0.1 or 1.0 mg/kg (equal to 0, 0.0003, 0.0023, and 0.0245 mg/kg bw per day) administered daily in two portions (half in the morning and half in the afternoon) for 42 days. Parameters assessed included mortality and clinical signs of toxicity, measurement of body-weight changes and food consumption, ophthalmoscopic examination, haematological and clinical chemistry determinations, urine analysis, and measurement of heart and respiratory rate. Necropsies were not conducted at study termination. Erythrocyte cholinesterase activity was determined before the start of treatment and on days 1, 3, 7, 14, 21 and 42. Erythrocytes were stored frozen for an unspecified period of time before analysis. Erythrocyte cholinesterase activity in groups treated with terbufos was compared with that for the appropriate concurrent controls at each time-point. Brain cholinesterase activity was not measured. The study report stated that the blood plasma of sheep had little or no cholinesterase activity and therefore was not assessed.

No deaths occurred during the study and no clinical signs of toxicity were observed. There was no obvious effect of treatment with terbufos on any parameter examined, including erythrocyte cholinesterase activity. It is not known whether storage conditions before assay had any effect on the cholinesterase activity of the erythrocytes. Brain cholinesterase activity was not assessed. The NOAEL in males (the only sex tested) was ≥ 1.00 mg/kg (equal to 0.0245 mg/kg bw per day) (the highest dose tested) (Garces et al., 1977). No statements of compliance with QA or GLP were provided. The study report stated that the facility in which the animals were maintained was fully accredited by the American Association for Accreditation of Laboratory Animal Care.

(b) *Exposure by inhalation*

In a short-term study of whole-body inhalation, groups of 10 male and 10 female Sprague-Dawley rats were given technical-grade terbufos (AC 92,100; purity, 90.1%) at a target concentration of 0, 0.005, 0.01, 0.05 or 0.10 mg/m³ (not adjusted for purity) as a vapour for 8 h per day, for 5 days per week over a period of 3 weeks, for a total of 15 exposures, followed by a 2-week recovery period. A 2-week treatment period had originally been planned, but during week 1 of exposure, analytical chamber concentrations were only about 2–44% of target concentrations. During exposure weeks 2 and 3, when vaporization flasks were heated slightly, analytical concentrations were much closer to those targeted, however, the range of daily means varied widely suggesting chamber concentrations were not well maintained. Mean daily analytical chamber concentrations and the range of daily means for exposure weeks 2 and 3 corresponding to the control, 0.005, 0.01, 0.05 and 0.10 mg/m³ groups were, respectively, for males 0, 0.0117 (range, 0.0003–0.0380), 0.0243 (range, 0.0066–0.05669), 0.0458 (range, 0.0098–0.0763) and 0.0946 (range, 0.0394–0.1523) mg/m³, and for females 0, 0.0112 (range, 0.0003–0.0380), 0.0256 (range, 0.0066–0.0569), 0.0468 (range, 0.0098–0.0763), and 0.1001 (range, 0.0448–0.01523) mg/m³. Estimated inhalation doses on a mg of active ingredient/kg bw per day basis for the control group and at the lower intermediate, higher intermediate and highest dose over the last 2 weeks of exposure, assuming a retained dose of inhaled material of 50%, were: males, 0, 0.0030, 0.0065, 0.0122 and 0.0246 mg/kg bw per day; and females, 0, 0.0041, 0.0097, 0.0175 and 0.039 mg/kg bw per day, according to the study report. Possible contributions to exposure via the dermal and oral routes (i.e. from grooming) were not discussed. Owing to mortality, exposure of females at the highest dose was stopped early, on day 18. The study was terminated for half the animals in all groups at the end of the exposure period on day 21 and for the other half at the end of the recovery period on day 37. Parameters evaluated for both the treatment and recovery phases of the study included observations for mortality, clinical signs of toxicity, measurement of body weight and food consumption, haematological and clinical chemistry determinations (in five animals of each sex per group), urine analysis, selected organ weight measurements (adrenals, heart, kidneys, liver, and lungs) and an examination for gross pathology. Plasma and erythrocyte cholinesterase activities were measured in five animals of each sex per group before testing, on days 1, 5, 8, 13 for all groups, on day 18 for surviving females at the highest dose and five control animals, and on days 21 and 37 for all groups. Brain cholinesterase activity was measured in five animals of each sex per group on days 21 and day 37. Cholinesterase inhibition was calculated relative to the appropriate concurrent control group.

All animals survived except for one male at 0.0117 mg/m³ that died accidentally on day 8 and two females at the highest dose that died on days 17 and 19 from treatment-related causes. The only clinical findings ascribed to treatment in the study were observed during the treatment period in the group of females at the highest dose and included body tremors, body coldness, and rapid or laboured breathing. Body weights were statistically significantly depressed in females at the highest dose, as was food consumption, particularly towards the end of the period of exposure. Haematological and clinical chemistry findings, which were generally statistically significantly different from the respective control group and which may have been related to treatment, were noted primarily during week 3. These included decreased haemoglobin, erythrocyte volume fraction and erythrocyte counts and an elevated clotting time in females at the highest dose, and decreased blood glucose concentrations in males at the highest dose. The results of urine analysis were not remarkable, although there were some low level increases in ketones, bilirubin and occult blood in males at the highest

dose at the end of the recovery period. The only remarkable organ weight change was a slight increase in the adrenal:body weight ratio of males at the highest dose (0.03580) relative to the control group (0.0272) at the end of week 3. No gross necropsy findings were considered to be related to treatment.

There were some inconsistencies observed in the data on cholinesterase activity, which sometimes made interpretation difficult, but it appeared that by the end of the exposure period (day 18 for females at the highest dose and day 21 for all the other groups), the following changes in blood cholinesterase activity were related to treatment: statistically significant inhibition of erythrocyte cholinesterase activity of 28% and 33% in females at the intermediate and highest doses; reduced erythrocyte cholinesterase activity of 22% in males at the highest dose with statistically significant reductions of 33% and 24% seen at the two previous time-points, respectively (days 13 and 8); statistically significant decreases in plasma cholinesterase activity of 30% and 61% in females at the intermediate and highest doses, respectively, and of 12% and 21%, respectively, in males at the intermediate and highest doses. Blood cholinesterase activity values were not statistically significant at the end of the recovery period. On day 21, brain cholinesterase activity was statistically significantly inhibited in females at the highest dose (based on data from three animals) by about 45% and in males by about 15% decreases that were considered to be associated with treatment (although the decrease in males was not considered to be toxicologically relevant). At the end of the recovery phase, brain enzyme activity values were not significantly different from those of controls, but statistically significant inhibition of 29% was observed in males at the highest dose, possibly indicating a lack of recovery. However, this interpretation of the data was questionable because statistically significant inhibition of 35% was also noted in brain cholinesterase activity at 0.0243 mg/m³, but not at the next highest dose of 0.048 mg/m³.

No other remarkable findings in other parameters, except as already noted, were reported for the recovery period. If plasma cholinesterase inhibition is not considered to be an adverse effect of treatment and toxicologically significant brain (not erythrocyte) cholinesterase inhibition and clinical signs of toxicity are considered to be relevant effects for terbufos, the NOAEL was 0.0458 (range, 0.0098–0.0763) mg/m³ (reflecting mean daily analytical chamber concentrations over the 3-week period of exposure and the range of daily means) in males and females on the basis of a statistically significant decrease and increase, respectively in blood glucose concentration and the adrenal to body weight ratio at the next highest dose in males, and mortality, clinical signs of toxicity, decreases in body weight and food consumption, and haematological changes in females at the next highest dose (Whitney, 1980). A statement of compliance with QA, but not for GLP, was provided. The protocol was not done to satisfy a particular guideline but was generally satisfactory for the intended purpose of the study.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a combined long-term study of toxicity and carcinogenicity, groups of 65 male and 65 female CD-1 mice were given technical-grade terbufos (AC 92,100; purity, 89.6%; prepared in a 1:1 solution of corn oil and methylene chloride) at a concentration (adjusted for purity) of 0 (vehicle), 3, 6, or 12 mg/kg equivalent to 0 (vehicle only), 0.45, 0.9 and 1.8 mg/kg bw per day for males and females for 18 months. Ten animals of each sex per group were scheduled for interim termination at 12 months (week 53) and the remaining

55 animals of each sex per group for termination at 18 months (week 80). The rationale for dose selection was not provided. Parameters assessed included observations for general health and mortality, clinical signs of toxicity, measurement of food consumption and body-weight changes, haematological determinations, organ weight measurements and macro- and microscopic pathology examinations. Cholinesterase activities were not assessed in this study.

Mortality was greater at the highest dose in both sexes relative to at the other doses at both interim and final termination. At week 53, mortality in the control group, and at the lowest, intermediate and highest dose was 3.6%, 0%, 1.8% and 12.7% for males, and 7.2%, 1.8%, 7.3% and 12.7% for females, respectively. At week 80, mortality in these same groups was 12.7%, 9%, 5.5% and 27.2% for males and 27.2%, 14.5%, 21.8% and 34.5% for females, respectively. A relationship to treatment for the decreased survival at the highest dose could not be dismissed (although the study report did not come to this conclusion). There were no clinical findings in the study that were obviously related to treatment. Body weight was statistically significantly decreased in both sexes relative to respective control groups at most weekly measurement time-points throughout the study. At week 54 and study termination, statistically significant reductions of about 8% were noted in males and of 10% and 11%, respectively, in females. At the intermediate dose, during the first 4–5 weeks of treatment, but not thereafter, statistically significant decreases in body weight of up to about 5% in males and up to about 8% in females were observed. These changes may have been related to treatment as they were not clearly associated with decreases in food consumption that might have indicated a palatability problem. Group mean body-weight gains over the entire study were reduced at the highest dose by 10% in males and 20% in females relative to respective control groups (statistical significance was not assessed) while group mean body-weight gains at the intermediate and lowest dose in both sexes exceeded those observed in animals in the control group. Statistically significant decreases in food consumption associated with treatment occurred at various times throughout the study at the highest dose in both sexes, with females somewhat more affected than males. Food consumption was not clearly affected by treatment at the intermediate and lowest doses. There were no remarkable findings in the haematological, organ weight, or gross pathology assessments for any group treated with terbufos. With regard to non-neoplastic findings noted microscopically, slight increases in total occurrences of fatty metamorphosis in the liver were observed in test material-treated groups of males and females. For males in the control group and at the lowest, intermediate and highest doses, respectively, the incidence was 13%, 13%, 25% and 28% and for females in these same groups, the incidence was 30%, 38%, 34% and 48%, respectively. In the absence of other associated findings (e.g. organ weights or other histopathological lesions), the increased instances were considered to be of a spontaneous nature and not related to treatment (there were no historical control data available for comparison). Other microscopic findings in the study had no obvious relationship with treatment. The NOAEL for systemic toxicity was 3 mg/kg, equivalent to 0.45 mg/kg bw per day in males and females, on the basis of statistically significant decreases in body weights in both sexes during the first 4–5 weeks of the study that were not clearly associated with decreased food consumption at the next highest dose. There was no evidence of carcinogenicity (Silverman et al., 1986). Statements of compliance with QA and GLP were provided. The protocol was consistent with US EPA Subdivision F Guidelines (November 1982 and revised, 1984).

Rats

In a combined long-term study of toxicity and carcinogenicity, groups of male and female Long Evans rats were given diets containing terbufos (AC 92,100; purity not specified) at a nominal concentration of 0.25, 1.00 or initially 2.00 mg/kg (nominally to 0.0125, 0.05, and 0.1 mg/kg bw per day) for 2 years. For both sexes, the highest dose was raised to 4 mg/kg (nominally 0.2 mg/kg bw per day) at the beginning of week 6 (day 35), raised again to 8 mg/kg (nominally 0.4 mg/kg bw per day) at the beginning of week 12 (day 77), and lowered again for females only to 4 mg/kg (nominally 0.2 mg/kg bw per day) during week 16 (around day 105). Two concurrent control groups of males and females were included in the study. Diets containing terbufos were prepared by adding a premix nominally containing 100 mg/kg of feed (0.01%) of active ingredient to lab chow to achieve the desired nominal concentrations. Diets were apparently not analysed for test material content, homogeneity or stability (no supporting data or information were provided in the study report), and intake of test material was estimated based on nominal dose and food consumption data. Animals in the control group were given lab chow only. The study was initiated with 60 animals of each sex per group. A subset of this group (five animals of each sex in the control group and 10 animals of each sex in each group treated with terbufos) was terminated at 3 months. The remaining 55 animals of each sex per group in the control group and 50 animals of each sex per group in each group treated with terbufos continued into the long-term portion of the study, which terminated at 24 months. During both parts of the study, all animals were monitored for mortality, clinical signs of toxicity, body-weight changes and food consumption. Also, during the 3- and 24-month portions of the study, urine analysis, and haematological and limited clinical chemistry examinations (serum glutamic-pyruvic transaminase, alkaline phosphatase, fasting glucose and blood urea nitrogen concentrations) were performed on three control animals of each sex per group and six animals of each sex in groups treated with terbufos. At study termination, a gross examination was performed on five control animals of each sex per group and on 10 animals of each sex in groups treated with terbufos at 3 months, on all survivors at 24 months and on all animals terminated in a moribund condition or found dead.

At 3 and 24 months, selected organ weights were measured (heart, kidney, liver and thyroid) in five control animals of each sex per group and on 10 animals of each sex in each group treated with terbufos and a histopathological examination was conducted on five control animals of each sex per group and on 10 animals of each sex treated with terbufos at the highest dose. Lung, liver, kidneys and heart were evaluated microscopically in a similar number of animals at the lowest and intermediate doses at 3 and 24 months. Tissues from other animals in the 24-month study were stored. Subsequent to the release of the original study report, tissues from all rats that were not previously processed were examined microscopically and the results of the 24 month exposure study were re-evaluated on this basis. Ophthalmoscopic examinations were performed only at 24-months. In three animals of each sex per control group and six animals of each sex in each group treated with terbufos, plasma and erythrocyte cholinesterase activities were determined for both sexes at 3, 6, 12 and 18 months, and brain cholinesterase activity was determined at 3 and 24 months. Inhibition of cholinesterase activity was assessed relative to values for the appropriate concurrent control groups.

There did not appear to be any deaths in the first 3 months of the study. At the intermediate dose, one animal, which was mis-sexed as a male, was placed with other females of that group, resulting in 59 males and 61 females. When the animals terminated at 3

months were not included in the calculations, mortality compared with that in the combined concurrent control groups was statistically significantly increased at the highest dose over the first 12 months of the study in males (28% in treated compared with 6.4% in the combined controls) and in females (28% compared with 0% in combined controls) and was statistically significantly increased over the 24 months at the intermediate and highest doses in males (57.1% and 62%, respectively, compared with 38.2% in combined controls) and in females at the highest dose (60% compared with 32.7%, respectively, in combined controls). Although a relationship of mortality rate and treatment could not be dismissed, the pathology report indicated illness existed among the test animals such as endemic bronchopneumonia (associated with bacterial infection) and pulmonary disease (said to be associated with the inhalation of food particles) and suggested that these conditions could have compromised animal well-being to a certain extent in some animals and/or contributed to the demise of others.

Neither individual nor summary data were provided in the study report for clinical findings or clinical signs of toxicity. According to the discussion in the study report, signs consistent with inhibition of cholinesterase activity (muscle tremors, excessive salivation, hyperactivity, hyperpnoea and tachycardia) were first noted in females at the highest dose during administration of the diet containing terbufos at 8 mg/kg (weeks 12–15). After the dose was lowered, the signs reportedly decreased in incidence in females at the highest dose and were not observed from months 18 until the end of the study. Some females at the intermediate dose reportedly exhibited some of the clinical signs (not specified) during months 5 and 6. Muscle tremors were reported in eight males at the highest dose during study months 22–24. Starting at around the time when females at the highest dose were placed on the die containing terbufos at 8 mg/kg, exophthalmos was noted in this group. Eventually, the condition manifested itself in all other groups of females including the controls and was said to persist until about month 15. The etiology of this condition was not clear. Ophthalmoscopic examination at the end of the study revealed an increase in corneal scarring and cataracts in males and females, but particularly in females, at the highest dose. Statistically significant decreases in body weight and food consumption compared with respective control values were observed in males and females at the highest dose throughout much or most of the 2-year study. At 24 months, there was no obvious effect of treatment on haematological, clinical chemistry or urine parameters examined. The significance, at 3 months, of small magnitude perturbations in concentrations of glucose and blood urea nitrogen in females at the highest dose was difficult to judge due to the changing doses. Small magnitude perturbations at the highest dose in relative or absolute weights at 3 months (kidney, heart, liver) and at 24 months (liver, kidney, and heart) may have been related to decreases in terminal body weights noted in this group.

Despite some variability in the magnitude and consistency of response among the various time-points measured, patterns of cholinesterase inhibition were noted for which an association with treatment could not be dismissed. Erythrocyte cholinesterase activity was statistically significantly inhibited at months 3, 6, 12, 18 and 24 at the highest dose in males (42–70%) and in females (35–80%). At the intermediate dose, statistically significant inhibition of erythrocyte cholinesterase activity was observed in males at months 6, 18 and 24 (32–40%) and in females at months 3 and 24 (43–46%). Brain cholinesterase activity was inhibited in males only at the highest dose at both 3 and 24 months (62–63%) and in females at the highest dose at 3 months (25%) and 24 months (58%) and marginally at the intermediate dose, 10% at 3 months (not statistically significant) and statistically significantly by 12% at 24 months. Inhibition of brain cholinesterase activity in females at the inter-

mediate dose was not considered to be toxicologically relevant. Plasma cholinesterase was statistically significantly inhibited in females at the highest dose at months 3, 6, 18 and 24 by 54 to 70% and marginally in males (statistically significant inhibition of about 40% only at months 12 and 18). Plasma cholinesterase data showed a remarkable degree of variability with time and dose.

Because an insufficient number of animals had originally been evaluated for pathology, a re-evaluation of the 24-month exposure period was conducted. Apparently, most of the tissues and masses from animals not previously processed for microscopic examination were available for examination. The total number of rats whose tissues were re-evaluated histopathologically out of the original numbers started on test (60) were for (males/females): control group I, 54/55; control group II, 53/55; at the lowest dose, 50/50; at the intermediate dose, 44/50; and at the highest dose, 47/49. Animals terminated after 3 months were not included.

A new gross examination could not be re-conducted, but the previous report was said to have been used, as far as was possible, to make correlations with regard to gross and microscopic examinations.

With regard to the microscopic examination, the re-evaluation report for the 24-month treatment period discussed a number of non-neoplastic findings in both males and females. Inflammatory lesions in the lung were associated with two conditions. One was the endemic bronchial pneumonia (thought to be related to bacterial infection) found in increased incidence in the group at the highest dose. There was also a higher incidence at the highest dose of a second type of pneumonia (granulomatous) attributed to the inhalation of food particles containing plant fibres which were said to act as foreign bodies but for which treatment with test material may have been a contributing or pre-disposing factor. It was suggested that an increased incidence of oesophageal distension in animals at the highest dose may have been related to an effect of treatment on muscle contractility, but the pathology report suggested possible relationship of this finding to the bacterial infection and sequelae of the bronchopneumonia. A higher incidence at the highest dose of gastric mucosal ulceration and/or erosion was also of uncertain etiology, although a relationship to treatment could not be dismissed.

The report concluded that there was no evidence that the test material had an effect on tumorigenesis.

The study was inadequate to assess chronic toxicity owing mainly to outstanding questions involving the etiology and/or relationship to treatment and/or dose of certain non-neoplastic findings (including ocular, lung and stomach lesions), and also uncertainty associated with the variability in some of the cholinesterase measurements for which a relationship to treatment could not be dismissed, and lack of sufficient documentation of clinical signs of toxicity. Therefore, a NOAEL for chronic (systemic) toxicity was not identified.

Despite some variability and inconsistencies, there were apparent response patterns noted in the data on cholinesterase activity for which an association with treatment could not be dismissed. If inhibition of plasma cholinesterase activity is not considered to be an adverse effect and toxicologically significant inhibition of brain (but not erythrocyte) cholinesterase activity is considered to be a relevant effect for terbufos, the NOAEL for cholinesterase inhibition was 1.00 mg/kg in males and females (nominally 0.05 mg/kg bw

per day) on the basis of statistically significantly decreased cholinesterase at the next highest dose.

With regard to the carcinogenicity phase of the study, information presented in the pathology re-evaluation report for the 24-month period of exposure was considered to be adequate to support the conclusion that there was no evidence of a carcinogenic response in the study, provided that the nominal intake of test material intake could be justified (Rapp, 1974). No statements of compliance with QA or GLP were provided.

2.4 Genotoxicity

The results of assays for genotoxicity with terbufos are summarized in Table 3.

Most of the tests for mutagenicity with terbufos in vitro and in vivo gave negative results. However, in one acceptably performed study of dominant lethal mutation in vivo, results were inconclusive. In a paper from the open scientific literature (Gentile et al., 1982), positive results were reported in an acceptably performed assay for mitotic gene conversion in yeast cells (*ade* locus) with technical-grade terbufos in the presence or absence of a metabolic activation system, and also with a commercial grade of terbufos, without metabolic activation. However, insufficient purity and analytical data were provided in the paper for the materials tested.

Although the results of an assay for unscheduled DNA repair synthesis in cells in primary culture were negative, only male Fischer 344 rat hepatocytes were used; an optimal protocol would also have included assessment of hepatocytes from female rats.

2.5 Reproductive toxicity

(a) Multigeneration study

Rats

In a two-generation study of reproductive toxicity, four groups of 25 male and 25 female Sprague-Dawley (COBS CD) rats, F₀ generation or F₁ generation parental animals, were given diets containing technical-grade terbufos (AC 92,100; purity, 89.6%; dissolved in a 1:1 solution of methylene chloride and corn oil) from the pre-mating period until termination of the adults. Terbufos was administered at a dietary concentration of 0 (vehicle only), 0.5, 1.0, or 2.5 mg/kg (equal to group mean intakes for the F₀ generation during the pre-mating period of 0, 0.035, 0.07, and 0.18 mg/kg bw per day in males, and 0, 0.04, 0.085, and 0.22 mg/kg bw per day in females, and for the F₁ generation during the pre-mating period of 0, 0.0372, 0.0742, and 0.1943 mg/kg bw per day in males, and 0, 0.04, 0.089, and 0.24 mg/kg bw per day in females. An additional group of F₀ generation animals was given terbufos at a dietary concentration of 5.0 mg/kg (equal to group mean intakes of 0.42 mg/kg bw per day in males and females); this group was, however, terminated early in week 6 of the pre-mating period because of toxicity in the females.

According to a standard protocol, the F₀ and F₁ generations were mated twice to produce F_{1a} and F_{1b}, and F_{2a} and F_{2b} litters, respectively. Dosing commenced in the F₀ generation at 63 days before mating when parental animals were aged about 7 weeks and continued for about 191 days in males and 210 days in females (during the lactation period for the F_{1b} litters). Initiation of dosing for F₁ generation parental animals (from F_{1b} litters) commenced after weaning of the F_{1b} litters and continued for about 205 days in males and

Table 3. Results of studies of genotoxicity with terbufos

End-point	Test object	Concentration/dose	Purity (%)	Results	Reference
<i>In vitro</i>					
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538; <i>E. coli</i> WP2 <i>uvrA</i> ⁻	50–5000 µg/plate, 1000 µg/disc in DMSO, ±S9 ^h	89.6	Negative	Allen (1985) ^{a,b}
Point mutation	Chinese hamster ovary cells (CHO- K ₁ -BH ₄), <i>Hprt</i> locus	10–100 µg/ml & in DMSO, ±S9 ^{c,i}	87.8	Negative ^d	Allen & Johnson, (1983) ^{a,b}
Chromosomal aberration	Chinese hamster ovary cells	2.5–100 nl/ml in DMSO, ±S9 ^{e,i}	87.8 ^f (1983) ^{a,b}	Negative	Thilager (1983)
Unscheduled DNA synthesis	Primary rat (male, Fischer 344) hepatocytes	0.33–33.33 µg/well in DMSO ^{g,i}	87.8 ^f	Negative	Godek (1983) ^{b,n}
Mitotic gene conversion	<i>S. cerevisiae</i> strain D4 (<i>ade</i> and <i>trp</i> loci)	Apparently 0.33–33 µg/tube in DMSO, ±S9	Technical and commercial grades (purities not specified; no analytical information)	Technical- grade: positive at the <i>ade</i> locus (±S9); Commercial-grade: weakly positive at the <i>ade</i> locus (–S9) ^o	Gentile et al. (1982)
<i>In vivo</i>					
Dominant lethal mutation (10 mating cycles)	Crl:CD(SD)BR rats (10 male rats per group)	0, 0.1, 0.2, or 0.4 mg/kg bw per day ^{h,j,k} in corn oil by gavage daily for 5 days at the start of the first mating cycle ^l	89.6	Inconclusive ^l	MacKenzie (1986) ^{a,b}
Chromosomal aberration	Sprague-Dawley rats (20 males, 20 females per group), bone-marrow cells	Single intraperitoneal doses of 0, 0.2, 0.6, 1.5 (females only) ^m or 1.8 mg/kg bw in corn oil ⁱ	89.6	Negative	Putnam (1986) ^{a,b}

DMSO, dimethyl sulfoxide

^aConsistent with US EPA Subdivision F guidelines (1984, revised)^bStatements of compliance with GLP and QA were provided^cCytotoxicity observed at 75 and 100 µg/ml in main study and at higher concentrations in the dose-finding study^dSlightly higher mutation frequencies (+S9) observed relative to the concurrent solvent control at 25 and 50 µg/ml were generally in the range of values for historical solvent controls and were attributed to unusually low mutation frequencies in concurrent controls. A repeat assay (+S9) gave negative results at concentrations of up to and including 50 µg/ml^eCytotoxicity was observed at 100 nl/ml in main study and at higher concentrations in dose-finding study^fPurity information obtained from the sponsor^gCytotoxicity observed at higher concentrations (100 µg/well and above)^hDose calculations corrected for % active ingredientⁱNo correction of dose for % active ingredient^jNo clinical signs of toxicity or effects on body weight were reported at the highest dose, but this dose was approximately one-tenth of the reported LD₅₀^kAfter treatment, each male was paired with two non-treated virgin females, 5 days per week for 10 weeks. Females were evaluated for number of implants, viable and nonviable fetuses and number of corpora lutea about 15 days after the mating period mid-point. Fertility index and implantation efficiency were calculated. The positive control used, triethylenemelamine, was administered intraperitoneally to 10 males for 5 days at a dose of 0.05 mg/5 ml saline/kg bw before the first mating cycle^lAt mating 9, the number of viable implants at 0.4 mg/kg bw per day was reduced slightly compared with the concurrent vehicle control group (statistically significant) and with respect to the positive control group (e.g. 12 compared with 14 and 13, respectively). At mating 7, implant efficiencies at the lowest, intermediate and highest doses (95%, 93%, and 89%, respectively) were lower (with a difference that was statistically significant at the lowest and highest doses) than that of the concurrent vehicle control group (98%) and the positive control group (97%). A slightly lower implantation efficiency (not statistically significant) relative to all other groups continued at the highest dose through the remaining three mating cycles numbers 8–10; the study report concluded that the NOAEL in males treated for 5 days was 0.2 mg/kg bw per day^mEvaluations of structural chromosomal aberration were conducted in five animals per group (including replacement animals) at 12, 24, and 36 h after dosing. Clinical signs of toxicity and mortality were observed after an intraperitoneal dose of 1.8 mg/kg bw in males and females and 1.5 mg/kg bw in females. Excessive mortality was observed in females at both of these doses, indicating an increased sensitivity of females to the test material. Protocol was consistent with US EPA Subdivision F guidelines (1984, revised), but only hepatocytes from males were used; hepatocytes from females should also have been assayed^oResults given only for the dose said to give highest recombinogenic activity. Assay protocol appeared to be generally acceptable, but no rationale for dose selection was presented. Commercial-grade terbufos gave negative results in *Zea mays* in a field plot assay for plant mutation in situ (technical grade not tested)

220 days in females. F_1 and F_2 litters were reduced to eight pups (equal numbers of each sex, if possible) on day 4 of lactation. F_{1a} , F_{2a} and F_{2b} litters were sacrificed at or after weaning on day 21 of lactation. Pups were examined externally for abnormalities and gross malformations. One F_{1b} and F_{2b} pup of each sex per litter, as well as any dead or stillborn pups, were necropsied and any abnormal tissues found were preserved.

Assessments performed for parental animals included observations for general condition, mortality and clinical signs of toxicity, measurement of body weight and food consumption, ophthalmoscopic examinations, evaluation of reproductive performance, including mating and fertility and pregnancy rate, duration of gestation and data on parturition, litter size and survival until day 21 of lactation, and macroscopic examination. Organ weight measurements were not made. At termination of each generation, histopathological evaluation was made of the following tissues: testes, epididymides, prostate, seminal vesicles, ovaries, uterus, vagina, pituitary and any gross lesions; this was performed only for animals receiving the highest dietary concentration (2.5 mg/kg) and for control F_0 and F_{1b} adults that had been selected for mating. Offspring parameters examined included assessment of general appearance, viability, litter sex ratio, and body weight (on days 1, 4, 7, 14, and 21 of lactation). Inhibition of plasma, erythrocyte and brain cholinesterase activities was calculated relative to the values for the appropriate concurrent control group for adult F_0 males and females (week 27) and F_{1b} adults, and for males and females at termination. Cholinesterase activity was not assessed in adult animals receiving terbufos at a dietary concentration of 5.0 mg/kg, nor in pups at any dose.

Six out of 25 females at the highest dietary concentration tested (5.0 mg/kg) died from treatment-related causes after 22–34 days. Clinical signs of toxicity, including tremors, a generally poor condition, emaciation, and unkempt appearance, were also noted in females in this group. There were no deaths among males at 5 mg/kg and few, if any, clinical signs were observed in males in this group during the same period (two males had excess salivation at pre-mating week 4). During certain periods before termination of all animals at 5.0 mg/kg at pre-mating week 6, statistically significant decreases in body weight were reported in both sexes, as were decreases in food consumption.

In the study of four groups, with 2.5 mg/kg as the highest dose, parental mortality was very low and of comparable incidence in groups treated with terbufos and respective control groups in the F_0 and F_1 generation. With the possible exception of soft stools in F_0 females at the highest dose during week 28 (12 occurrences compared with 2, 3 and 6 occurrences in the control group, and at the lowest and intermediate doses, respectively), there were no clinical findings that could definitively be associated with treatment with terbufos in either the F_0 or F_1 parents. Animals with symptoms considered in the study report to be consistent with sialodacryoadenitis viral infection (including dry eyes in females at the lowest and intermediate doses) were noticed during weeks 26 to 28 (towards the end of the F_0 generation). The study report indicated that the outcome of the study was considered not to have been affected by the presence of the virus at this stage.

There was no apparent effect of treatment on male or female body weight of parents in either the F_0 or F_1 generations, except in lactating females at 2.5 mg/kg. Over the duration of the lactation period (days 0–20) for F_{1a} , F_{1b} , and F_{2b} litters, females at the highest dose exhibited statistically significant body-weight loss relative to that in the respective control groups. Food consumption did not appear to be adversely affected in parental

animals of either generation. The results of ophthalmoscopic examinations were not remarkable.

In F_0 adults, statistically significant inhibition of plasma cholinesterase activity was observed in males at the highest dose (46%) and females at the intermediate and highest doses (61% and 94%, respectively). Statistically significant reductions were noted in erythrocyte cholinesterase activity in males at the highest dose (11%) and in females at the intermediate and highest doses (7% and 15%, respectively). Brain cholinesterase activity was statistically significantly inhibited in males at 1.0 and 2.5 mg/kg (8% and 29%, respectively) and in females at 0.5, 1.0, and 2.5 mg/kg (7%, 22%, and 66%, respectively).

In F_1 adults, statistically significant inhibition of plasma cholinesterase activity was observed in males (by 20% and 53%, respectively), and in females (by 50% and 87%, respectively) at the intermediate and highest doses. Erythrocyte cholinesterase activity was statistically significantly reduced only at the highest dose in males (by 15%) and in females (by 13%). Brain cholinesterase activity was statistically significantly inhibited at the intermediate and highest doses in males (by 8% and 34%, respectively) and in females (by 21% and 59%, respectively).

Decreases in brain cholinesterase activity of 7–8% in F_0 and F_1 adults at the lowest and intermediate doses, although possibly treatment-related, were not considered to be toxicologically significant.

At 2.5 mg/kg, a treatment-related reduction in male fertility and the number of pregnant females was observed for the production of F_{1b} litters and F_{2b} litters compared with values for these parameters in the respective control groups. In the control group, and at the lowest, intermediate and highest dose, pregnancy rates were 92%, 88%, 96% and 80% in the first generation (F_{1b}) and 86%, 92%, 82% and 63% in the second generation (F_{2b}), respectively. The corresponding male fertility index (number impregnating per number mated) for F_{1b} litters was 91%, 87%, 96% and 79% and for F_{2b} litters, 94%, 95%, 94% and 61%, respectively.

At the highest dose, there were some, generally slight, changes in several F_{1b} and F_{2a} and F_{2b} neonatal parameters (such as in mean numbers of pups, live pups or dead pups per litter and total numbers of live or dead pups per litter). When these parameters were looked at overall, they were suggestive of possible subtle treatment related decreases in pup viability, number and/or survival at 2.5 mg/kg. However, they lacked statistical significance or persistence; the only statistically significant change, a decrease in the number of live offspring in F_{1a} litters relative to that in the control group (9.8 compared with 12.8) before reduction of litter sizes on lactation day 4, was not found at later time-points).

A more concrete case for an effect of treatment with terbufos on offspring concerned pup body weights. On days 14 and 21 of lactation, decreases (about 15–17%) in the mean weight of viable pups relative to those of pups in the the respective control group, were observed in F_{1a} , F_{1b} , F_{2a} (smaller decreases of 7–9%) and F_{2b} litters and were considered to be related to treatment. Decreases were statistically significant in F_{1a} litters, F_{1b} litters (a decrease of 11% was also noted on day 7), and F_{2b} litters (a decrease of 16 % was also noted on day 7).

Other parental reproductive or neonatal parameters examined were not obviously affected by treatment with terbufos. There were no obvious treatment-related findings upon gross examination of pups or adults, or upon histopathological evaluation of the adult tissues and organs selected for analysis.

The NOAEL for reproductive effects was 1.0 mg/kg for males and females (equal to 0.07–0.074 mg/kg bw per day in males and 0.0854–0.089 mg/kg bw per day in females) on the basis of decreases in male fertility and pregnancy rate, respectively. The NOAEL for parental toxicity was 2.5 mg/kg in males (equal to 0.18–0.19 mg/kg bw per day) on the basis of statistically significant decreases in body weight and decreases in food consumption at the next highest dose and 1.0 mg/kg in females (equal to 0.085–0.089 mg/kg bw per day) on the basis of statistically significant body-weight loss during lactation and an apparent increase in soft stools at the next highest dose. The NOAEL for offspring toxicity was 1.0 mg/kg (equal to 0.07–0.074 mg/kg bw per day in males and 0.085–0.089 mg/kg bw per day in females) on the basis of statistically significant decreases in the mean body weight of viable pups on days 14 and 21 of lactation in F₀ and F₁ litters.

If plasma cholinesterase inhibition is not considered to be an adverse effect and toxicologically significant brain (not erythrocyte) cholinesterase inhibition is considered to be a relevant effect for terbufos, the NOAEL for cholinesterase inhibition was 1.0 mg/kg (equal to 0.07–0.074 mg/kg bw per day) in parental males and 0.5 mg/kg (equal to 0.04–0.044 mg/kg bw per day) in parental females on the basis of statistically significant decreases in brain cholinesterase activity in the F₀ and F₁ generations at the next highest doses (Schroeder, 1989). Statements of compliance with QA and GLP were provided. The protocol was consistent with US EPA Subdivision F guidelines (1982 and 1984, revised).

(b) Developmental toxicity

Rats

In a study of developmental toxicity, groups of Charles River albino rats were given technical-grade terbufos (AC 92,100; purity not specified) at a dose of 0 (vehicle only; 20 rats), 0.075 (20 rats) or 0.150 (21 rats) mg/kg bw per day daily on days 6–15 of gestation, inclusive, by gavage in corn oil. The study was terminated on day 20 of gestation. The basis for dose selection was not discussed. Adult animals were observed for mortality, unusual reactions, clinical signs of toxicity, and body-weight changes. Other parameters assessed were group mean numbers of corpora lutea, implantation sites, resorption sites, and viable fetuses, as well as number of fetuses, group mean fetal body weights, sex ratio and the incidence of females with one or more resorption sites. Fetuses were examined externally for effects of treatment and, where possible, approximately equal numbers of each sex were evaluated for skeletal and internal developmental abnormalities using the Hurley method of staining with alizarin, or the Wilson and Warkany technique, respectively. Cholinesterase activity was not measured.

There were no deaths or unusual reactions in adult animals. No treatment-related effects were noted in any parameter. This study had many deficiencies, including the following: the purity of the test material and the concentration of the test material in dosing solutions could not be confirmed; it was not clear if randomization techniques were used in assigning animals to treatment groups; no data for individual adult animals or fetuses were provided in the study report; the number of dead fetuses was not indicated; maternal or fetal data were generally reported only as group means; it was not clear how fetuses were

selected for examinations for developmental abnormality; fetal abnormalities were not assessed on a litter basis; the number of fetuses examined for abnormalities did not match the number of fetuses reported; complete necropsies of adult animals were not conducted. Also, animals were shipped to the laboratory on day 1 of gestation and were allowed no acclimation period. NOAELs for maternal and developmental toxicity could not be identified owing to the many deficiencies in study design and data reporting (Haley, 1972). No statements of compliance with GLP or QA were provided, the protocol was not consistent with EPA or OECD guidelines and was unacceptable by current standards; the study was conducted in 1972 (before the establishment of guidelines for GLP and EPA guidelines).

In a preliminary study of developmental toxicity, groups of five female Charles River COBS® CD® rats were given technical-grade terbufos (AC 92,100; purity, 87.8%) at a dose (adjusted for purity) of 0 (vehicle only), 0.4, 0.8, 1.4, 3, or 6 mg/kg bw per day once daily by gavage in corn oil during days 6–15 of gestation, inclusive. These doses were actually twice the amounts intended owing to an unintentional protocol error such that the volume administered was 10 ml/kg instead of 5 ml/kg. Owing to excessive toxicity and mortality in all groups treated with terbufos, the study was terminated prematurely (before day 20 of gestation) and two additional groups of five females were subsequently given the test material at a dose of 0.05 or 0.2 mg/kg bw per day administered in a volume of 5 ml/kg. This second phase was terminated as planned on day 20 of gestation. For all groups in both phases, planned evaluations in adult females included observations for general condition and clinical signs of toxicity, mortality and moribundity, body-weight changes, abortions, number of corpora lutea, and terminal necropsy. Litter and fetal assessments included number of viable and dead fetuses and fetal position in the uterus, number of implantation sites, early and late resorptions, early implant loss in uteri with no gross evidence of implantation, and postimplantation loss. No examinations were conducted of fetuses for external, visceral or skeletal abnormalities, body weight or sex ratio in either phase and cholinesterase activity was not measured.

During the first phase of the study, all animals died or were sacrificed in a moribund condition as a result of treatment at doses of ≥ 0.4 mg/kg bw per day. The cause of death was cardiorespiratory arrest or cerebral haemorrhage. Findings at necropsy included hepatic congestion, renal and pulmonic hyperaemia, gastric congestion and loss of epithelium, intestinal congestion and diarrhoea, and in some animals at doses of 1.4 mg/kg per day and above, haemorrhagic intestines. Deaths at 0.4 mg/kg bw per day occurred between days 10 and 16 of gestation. At higher doses, deaths or early terminations occurred on days 7–9 of gestation. Severe body-weight losses were observed in all groups treated with the test material. Clinical signs of toxicity, described as primarily yellow urogenital matting and dried red matter around the eyes and nares were noted primarily in animals given doses of 0.4 and 0.8 mg/kg per day, as survival was longest in these groups. Pregnancy rates in animals in the vehicle control group and groups treated with terbufos ranged from 80% to 100%. No abortions were reported. Uterine assessments were incomplete owing to mortality but, relative to groups receiving the highest dose, an increase in early litter resorptions was noted at necropsy at doses of 0.4 mg/kg bw per day and, to a lesser extent, at 0.8 mg/kg bw per day; this effect may have been related to maternal toxicity and day of gestation. At 0.4 mg/kg bw per day and 0.8 mg/kg bw per day, respectively, early resorptions were observed in five out of five gravid females (maternal deaths on days 10–15 of gestation in this group) and two out of four gravid females (maternal deaths on days 8 or 9 of gestation in this group), while none were reported in pregnant animals at doses of ≥ 1.4 mg/kg bw per day (maternal deaths on day 7 of gestation in these groups).

In the second phase of the study, the results provided indicated that doses could have, at least sometimes, been 10–15% below nominal levels. No mortality or clinical signs of toxicity were reported at 0.2 or 0.05 mg/kg bw per day. Groups treated with terbufos gained slightly less weight than did the vehicle control group during dosing (days 6–16 of gestation) and overall gestation (days 0–20 of gestation), but a statistical analysis of the data was not performed. Pregnancy rates were 100%, 80% and 100% in the vehicle control group and groups receiving the lowest and intermediate doses, respectively. A delivery early in the study on day 10 of gestation by one female at the lowest dose was attributed to an error in the detection of mating, such that the day of parturition was assumed to be 22 on the basis of pup size and development. The results of necropsy of this and other animals in the second phase were not remarkable. There were no other premature deliveries nor were there any abortions. Litter incidence (relative to that in the vehicle control group) of viable fetuses, implantation sites and corpora lutea were similar for the three dams available for evaluation at the lowest dose and the five available at the highest dose. There were no dead fetuses or late resorptions in any group. A slightly higher postimplantation rate of 1.7 per litter at 0.05 mg/kg bw per day, associated with early resorptions, was noted compared with a value of 1.0 per litter in the control group, but the value for the group receiving the lowest dose was based on an evaluation of three animals only and the rate at 0.2 mg/kg bw per day (0.6 per litter) was lower than that for the control group. On the basis of the parameters assessed in phases one and two, the NOAEL for maternal toxicity was 0.2 mg/kg bw per day, as doses administered above this were excessively toxic. A NOAEL for developmental toxicity could not be identified owing to the small number of animals evaluated and the limited study design and assessments made (Rodwell, 1984). Statements of compliance with QA and GLP were provided. Protocol deficiencies were noted, but the study was not performed to meet a specific guideline, being a preliminary study.

In a study of developmental toxicity, groups of 25 Charles River COBS CD rats were given technical-grade terbufos (AC 92,100; purity, 87.8%) at a dose of 0 (vehicle only), 0.05, 0.10, or 0.20 mg/kg bw per day by gavage in corn oil once daily on days 6–15 of gestation, inclusive. Doses were adjusted for test material purity. The study was terminated on day 20 of gestation. Selection of doses for this main study was based on the results of a preliminary study of developmental toxicity in the same strain of rats; treatment-related mortality and clinical signs of toxicity had been reported after administration of test material at daily doses of ≥ 0.4 mg/kg bw per day by gavage on days 6–15 of gestation, inclusive (Rodwell, 1984). In the main study, evaluations in adult females included observations for general condition and clinical signs of toxicity, mortality and moribundity, body-weight changes, abortions, number of corpora lutea, and terminal necropsy. Litter and fetal assessments included number of viable and dead fetuses, number of implantation sites, early and late resorptions, early implant loss in uteri with no gross evidence of implantation, postimplantation loss, and fetal weight, sex, sex ratio, and uterine location. All fetuses were examined for external abnormalities. Approximately one-half of the fetuses from each litter were examined for soft tissue findings using the Wilson sectioning method, while the remainder were examined for skeletal defects using a modification of the Dawson alizarin red S staining technique and low power magnification. Cholinesterase activity was not measured.

In adult animals, no mortalities or treatment-related clinical signs of toxicity were observed during the study. Slightly lower body-weight gain was noted in groups treated with terbufos at the intermediate and highest doses, relative to the control group, during dosing (days 12–16 of gestation and during days 6–16 of gestation), and after dosing (days 16–20

of gestation). During days 12–16 of gestation, body-weight gains at the intermediate and highest doses were 7% and 10% less than that of the control group, respectively, while during days 6–16 of gestation, body-weight gains in these groups were 7% less than that of the control group. After dosing, body-weight gains at the intermediate and highest doses were 5% less than that of the control group. In the study report, the relatively lower body-weight gains at the intermediate and highest doses were considered to be related to administration of the test material. These decreases in body weight were not considered to be toxicologically significant, being of similar magnitude at the intermediate and highest doses, relatively small, and not statistically significant. In addition, there was no indication of an increase in body-weight gain after cessation of dosing.

Pregnancy rates were similar among all groups, ranging from 96% to 100%, and there were no abortions or premature deliveries.

There were no statistically significant differences in the number of corpora lutea or implantation sites in groups treated with terbufos relative to values for these parameters in the concurrent control group. Late resorptions, although not statistically significantly increased, were observed only at 0.2 mg/kg bw per day. All late resorptions (eight) and the only dead fetus reported in the study were found in the litter of one dam at the highest dose, along with one early resorption and one fetus with no remarkable findings out of a total of 11 conceptuses. At necropsy of the dam (which survived the study), abnormalities described collectively in the study report as “severe pathology” but not treatment-related, were found on gross examination of the liver (described as pale and soft with accentuation of lobular markings), spleen (described as congested enlarged and indurated) and kidney (described as pale, bilateral). Follow-up histopathology was not performed. Weight gain (50 g) in this animal during dosing (days 6–16 of gestation) was not dramatically different from the mean for the control group (57 g) or the mean for the group receiving the highest dose (53 g); however, it was the only animal at the highest dose to lose weight (–1 g) after cessation of dosing (mean weight gain at the highest dose after cessation of dosing was 60 g). The relationship between litter/fetal findings and the pathology observed in this animal is not clear.

Early resorptions at the intermediate and highest doses of 1.2 and 1.0 per litter, respectively, were slightly elevated (not statistically significantly) relative to values for the concurrent control group (0.8 per litter) and the group receiving the lowest dose (0.5 per litter).

Fetal body weights were comparable in all groups. The number of viable fetuses at the intermediate and highest doses (13.7 and 13.6 per litter, respectively) were slightly lower, although not statistically significantly so, than those in the concurrent control group and the group receiving the lowest dose (15.0 and 14.8 per litter, respectively), and were within the range of data for historical controls for the laboratory and rodent strain used (mean, 13.9 per litter; range, 11.9–15.4). These decreases in the number of viable fetuses were attributed to slight increases (not statistically significant) in postimplantation loss at the intermediate and highest doses (1.2 and 1.3 per litter, respectively) compared with those in the respective concurrent control group and group receiving the lowest dose (0.8 and 0.5 per litter, respectively). At the intermediate dose, the increased postimplantation loss (1.2 per litter) was just inside the range for historical controls (mean, 0.6 per litter; range, 0.1–1.2) and was associated with losses due to early resorption (no early resorption data for historical controls were provided). At the highest dose, postimplantation loss (1.3 per litter) was just outside the range for historical controls and was associated with both early and late resorptions and the one fetal death. If litter/fetal data from the dam with the reported macro-

scopic pathology at necropsy were not considered, the changes in litter or fetal parameters at the highest dose relative to those in the control group appeared to be eliminated.

There was a statistically significant difference in the group mean fetal sex ratio in all groups treated with terbufos relative to the value for concurrent controls. This was attributed in the study report to a skewed sex ratio in the concurrent control group (male:female, about 1:1.4) relative to that based on the historical control data provided (calculated as male:female 1:1.006), although group means and ranges for historical controls were not provided in the study report. External, visceral and skeletal fetal examinations did not reveal any morphological findings associated with treatment.

The study authors did not consider any of the findings on fetal viability, resorptions, or postimplantation loss at the intermediate and highest doses to be biologically meaningful, because none of the changes were statistically significant relative to the control group. Available data for historical controls generally supported this contention at the intermediate dose and, in the case of fetal viability, at the highest dose. In addition, there was no apparent effect on postimplantation loss, fetal viability or resorptions (early or late) at the highest dose, if litter/fetal data from the female with reported pathology at the highest dose are eliminated from consideration. Although no clear maternal or developmental toxicity was considered to have occurred in this main study, mortality was seen at a dose of 0.4 mg/kg bw per day in a preliminary study (Rodwell, 1984), at just twice the highest dose used in the main study (0.2 mg/kg bw per day), thus providing support for dose selection in the main study.

The NOAEL for maternal toxicity and developmental effects was 0.2 mg/kg bw per day, the highest dose tested (Rodwell, 1985). Statements of compliance with GLP and QA were provided and the protocol was consistent with US EPA Subdivision F (1982) guidelines.

Rabbits

In a study of developmental toxicity, groups of 17 New Zealand white (Hra:(NZW)SPF) rabbits were given technical-grade terbufos (AC 92,100; purity, 89.6%) at a dose (adjusted for purity) of 0 (vehicle only), 0.05, 0.10, 0.25 or 0.50 mg/kg bw per day by gavage in corn oil, once daily during days 7–19 of gestation. The study was terminated on day 29 of gestation. Data on dose selection were not provided. Upon analysis, the range of received concentrations of test material was 87–98% of target concentrations. Evaluations in adult females included observations for general condition and clinical signs of toxicity, mortality and moribundity, body-weight changes, food consumption, abortions, premature delivery, uterine weights, number of corpora lutea, and terminal necropsy. Litter and fetal assessments included number of viable and dead fetuses, number of implantation sites, early and late resorptions, postimplantation loss, and fetal weight, sex, sex ratio and uterine location. All fetuses carried to study termination were examined for external, visceral and skeletal abnormalities using standard techniques (including the use of alizarin red S staining). Cholinesterase activity was not measured.

There were no adult deaths and no premature deliveries. Two animals aborted, one in the group receiving the highest dose on day 21 of gestation (9 conceptuses) and the other in the group receiving a dose of 0.10 mg/kg bw per day (6 conceptuses) on day 22 of gestation. Litters were not available for analysis, presumably due to cannibalization. The

study report did not attribute this litter loss to treatment, as there were no signs of toxicity or apparent gross lesions at necropsy. There was some decrease in body weight and food consumption in both animals in the days immediately before abortion. A possible relationship of abortion at the highest dose to treatment could not be discounted, as other maternal treatment-related findings occurred at that dose.

The only clinical related to treatment was a statistically significant increase in the incidence of does with soft or liquid faeces at a dose of 0.5 mg/kg bw per day. This effect, which did not occur in the vehicle control group, was observed in four rabbits on 1 or 2 days and was associated with some decrease in body weight and food consumption, but no gross lesions at necropsy. Three rabbits (one not pregnant) had occurrences during the latter part of or just outside of the dosing period during days 16–20 of gestation. Another pregnant animal had one occurrence on day 29 of gestation. Instances of soft or liquid faeces associated with transient decreases in body weight and food consumption at other doses were not considered to be related to treatment, as there was no statistically significant increase and the incidence was low (e.g. found once, on day 18, in one animal at 0.05 mg/kg per day and twice, on days 24 and 25, in one animal at 0.10 mg/kg bw per day).

Maternal body-weight gain at the two higher doses (+ 0.16 and +0.16 kg, respectively) was decreased relative to that in the vehicle control group and at the two lower doses (+0.26, +0.23 and +0.23 kg, respectively) during the time interval encompassing the dosing period (days 7–20 of gestation). Between days 16 and 20 of gestation, there was some loss of body weight in animals at the highest dose, which was not seen in other groups. Although none of the body-weight findings at the two higher doses was statistically significant, in the study report they were considered to be related to treatment. Slight decreases in food consumption relative to that of controls were only noted at the two higher doses and then only during the period after cessation of dosing (days 20–29 of gestation); the study report suggested that this could have been related to a delayed effect of treatment. The present reviewer considered that his interpretation was possible but questionable. At necropsy, two animals at the highest dose (not those with soft or liquid faeces) were found to have reddened areas in the fundic area of the stomach that were considered to be treatment-related in the study report. The results of necropsy in other animals were not remarkable.

The incidence of pregnancy in the control group and at 0.05, 0.10, 0.25 and 0.50 mg/kg bw per day was 94%, 71%, 82%, 76% and 88%, respectively. There was no apparent effect of treatment with the test material on the number of corpora lutea, implantations, or live and dead fetuses (all fetuses were reported to be alive at study termination), or on litter size, sex ratio, or uterine weights (not including data from the two females that aborted). Although incidences of early or late resorptions were comparable between groups, at the highest dose there was an increase in the incidence of does with any resorption site. For the control group and at 0.05, 0.10, 0.25 and 0.50 mg/kg bw per day, the total number (and percentage) of does with resorptions of any type was 3 (18.8%), 5 (41.7%), 5 (38.5%), 4 (30.8%) and 10 (71.4%), respectively. The increase at the highest dose was not statistically significant and was not considered to be treatment-related in the study report. However, the incidence was higher than that in the concurrent control and was outside of the range for historical controls (data provided on a percentage basis, e.g. mean, 129 (39.2%) with a range of 0 (0%) to 10 (64.3%) based on data from 329 control groups from 21 studies conducted at the test facility between 1986 and 1988; current study conducted in 1988), such that a relationship to treatment could not be dismissed. In addition, a slight decrease in the group mean fetal body-weight at the highest dose (42.48 g) relative to that of the

concurrent control (44.8 g) was considered by the study authors to be an effect of treatment, although the difference was not statistically significant. Similar values were obtained when male and female fetal body weights at the highest dose were separately compared with those for the appropriate concurrent control group. No fetal external, visceral or skeletal findings were considered to be related to treatment with the test material.

With regard to maternal toxicity, although the study report suggested that there was a treatment-related effect at the intermediate dose, the evidence cited to support this contention (a relatively small body-weight decrease during the dosing period, which was not dose-dependent and was not statistically significant in a species in which body-weight variability is common; and possibly a slight decrease in food consumption after dosing) is of questionable toxicological significance in the absence of other findings. The NOAEL for maternal toxicity was 0.25 mg/kg bw per day on the basis of an increased number of does with soft or liquid faeces, maternal body-weight loss during days 16–20 of gestation, occurrence of reddened areas in the fundic region of the stomach at necropsy, and one abortion at the next highest dose. The NOAEL was 0.25 mg/kg bw per day on the basis of decreased fetal body weights and an increase in the incidence of does with any resorption sites at the next highest dose (Hoberman, 1988a, 1988b). Statements of compliance with GLP and QA were provided and the protocol was consistent with EPA Subdivision F Guidelines (1982 or 1984, revised).

2.6 *Special studies*

(a) *Neurotoxicity*

(i) *Acute delayed neurotoxicity*

Technical-grade terbufos (AC 92,100; purity, 96.7%) was tested for acute delayed neurotoxicity potential in sex-link hens (aged approximately 1 year). Confirmation of the content of test material in the dosing solution was not provided in the study report. In a preliminary study to aid in dose selection for the main study, the acute oral LD₅₀ in this strain of hen for technical-grade terbufos in corn oil was estimated by probit analysis to be 40 mg/kg bw (95% confidence interval (CI), 31.8–48.2 mg/kg bw). The test material was administered by gavage as single doses at 10 (one animal), 20 (one animal), 28.3 (three animals), 40 (four animals), 56.6 (four animals) or 80 mg/kg bw (two animals). The incidence of mortality in these groups respectively, was 0/1, 0/1, 1/3, 1/4, 4/4 and 2/2. There was some variability in the time to death. Mortality was observed at 22 h after dosing at 28.3 mg/kg bw, at 4.5 h after dosing at 40 mg/kg bw, at 1.5–22 h after dosing at 56.6 mg/kg bw and within 2.5 h of dosing at 80 mg/kg bw. The only clinical findings reported were observed in some animals at 28.3 and 40 mg/kg bw and consisted of wobbly gait or unsteady gait and stance at 20–24 h after dosing, and resolved by 70–72 h after treatment.

The main study consisted of two phases. In phase one, 10 hens received the test material in corn oil as a single dose of 40 mg/kg bw by gavage. Included in this group of 10 were the three surviving animals that had received a dose of 40 mg/kg bw in the preliminary study for determination of acute LD₅₀. Other groups of hens received corn oil alone (four animals) or tri-ortho-cresyl phosphate (TOCP) at 500 mg/kg bw in corn oil as the positive control (10 animals). There was no pretreatment with atropine. Hens were observed for 21 days after dosing for mortality, signs of neurotoxicity and locomotor abnormalities.

All animals surviving study phase one were entered into study phase two. This inadvertently included any animals exhibiting effects consistent with delayed neurotoxicity (e.g. animals treated with TOCP) that were originally intended for sacrifice on day 22 after dosing in phase one. In phase two (which appeared to commence about 26 days after phase one dosing), the seven terbufos-treated hens surviving phase one received a second single dose of the test material by gavage in corn oil, while the surviving three or seven hens in the vehicle and positive control groups, respectively, were similarly dosed a second time with either corn oil only or 500 mg/kg bw of TOCP. Animals treated with TOCP were pretreated with atropine. After a 21-day observation period, animals treated with TOCP (seven) and vehicle control group animals (three) were terminated on day 22 after dosing. Surviving hens (six) treated with TOCP were terminated three days after dosing in phase two (i.e. 29 days after dosing in phase one). The original study report indicated that the spinal cords of only the three animals in the vehicle control group and the six animals treated with TOCP (animals considered to display signs of (delayed) neurotoxicity) were subjected to histopathological examination. Procedures used in preparation for the examination were only described briefly in the study report. It was stated that spinal cords were fixed *in situ* (perfusion was not indicated) in buffered formalin for 2 days before sectioning. Although brain, spinal cord (cervical, thoracic and lumbosacral sections), and sciatic nerve tissue were taken, only lumbosacral spinal cord sections (stained with haematoxylin and eosin) were examined microscopically. The tibial nerve was not examined. The hens were not subjected to a period of forced motor activity and there was no evaluation of cholinesterase or neuropathy target esterase activities in either study phase one or two.

During phase one of the main study, one out of four control animals died on day 19 and exhibited no signs or symptoms before death. Three out of 10 animals treated with terbufos and not pretreated with atropine died; two on day 0 and one on day 12 after dosing. No clinical symptoms were reported for the animals that died on day 0. The animal that died on day 12 exhibited two instances of wobbly gait and/or wobbly stance of low severity on days 1 and 7 after dosing. On day 1 after dosing, three additional animals treated with terbufos displayed single occurrences of low severity wobbly gait and/or wobbly stance. The deaths and clinical observations in the animals receiving terbufos were attributed to cholinergic toxicity. None of the animals treated with terbufos or the vehicle control animals dying during phase one were examined histopathologically. Based on the deaths in the terbufos animals in the preliminary study and in phase one of the main study, the acute oral LD₅₀ in hens was re-estimated to be 43.5 mg/kg bw.

Animals treated with TOCP in phase one exhibited no signs of acute toxicity but did have symptoms of locomotor impairment (wobbly gait and/or wobbly stance) that started on day 11 after dosing in all animals and grew progressively worse, such that by day 21 after dosing most animals fell while walking, could only stand or walk in a squatted position or could not walk or stand at all. Three of the most severely affected hens died 26 days after dosing in phase one and were not examined histopathologically.

After dosing in phase two, signs of acute toxicity were observed in all seven animals treated with terbufos (and pretreated with atropine). The findings, which were attributed to cholinergic toxicity, lasted up to 72 h and were generally of low severity (wobbly gait and/or wobbly stance) except in the case of one animal that could not walk or stand one day after dosing only. There were no observational findings in these animals after day 3 after dosing. According to the study report, these animals were not examined histopathologically after

termination on day 22 after dosing in phase two because they showed no indications of delayed neuropathy.

Of the seven hens that inadvertently received a second dose of TOCP, three (which already had severe symptoms of locomotor impairment subsequent to phase one) died on day 2 or 3. The remaining hens in this group were terminated on day 3 after dosing in phase two and the spinal cord of six of the seven hens was subjected to histopathological examination. Minimal (two hens), slight (two hens), and moderate (two hens) degrees of demyelination of spinal cord fibre tracts (white matter) were found. One animal had swollen axis cylinders (white matter) and another had gliosis, both effects being of low severity. There were no observational or histopathological findings in the three control animals carried over into phase two.

Subsequently, the spinal cords of the seven animals treated with terbufos in phase two were examined histopathologically; it is not clear how the tissues of these animals were prepared for examination. Two animals had a perivascular lymphocytic cell infiltrate of low severity in the spinal cord, which was not considered to be of any concern. No demyelination or other adverse changes were apparent in the tissues examined. There was no evidence to suggest that terbufos caused delayed neuropathy in hens under the conditions of the study (Smith, 1972, 1973). No statements of compliance with QA or GLP were provided. The protocol was generally consistent with EPA Subdivision F Guidelines (1982 and 1984, revised).

(b) *Acute neurotoxicity*

In an study of acute oral neurotoxicity, groups of 20 male and 20 female Sprague-Dawley-derived (outbred) Crl:CD®(SD)IGS BR VAF/Plus® rats (fasted before dosing) were given technical-grade terbufos (AC 92,100; purity, 89.7%) as a single dose (not adjusted for purity) of 0 (vehicle only) 0.15, 0.30, or 0.90mg/kgbw by gavage in corn oil. The study was terminated on day 15, following an observation period after dosing.

The selection of doses administered in the definitive study was based on the results of a pilot study to determine time of peak effect in males and females of the same strain of rat. In this study, groups of five males and five females were given single doses of the test material at (0 (vehicle only), 0.025, 0.05, 0.15, 0.40, 1.2 or 1.6mg/kgbw in males and 0 (vehicle only), 0.025, 0.05, 0.15, 0.30, 0.90 and 1.2mg/kgbw in females by gavage in corn oil. Five additional females were treated with terbufos at the highest dose, in case replacements were needed. All animals were monitored for clinical signs of toxicity, and at about 6h after dosing (designated as the time of peak effect), plasma, erythrocyte and brain cholinesterase activities were measured in five animals of each sex per group. In the pilot study, one female died from causes attributed to treatment with the test material within 5h of treatment with a dose of 1.2mg/kgbw. Clinical signs of toxicity consistent with inhibition of cholinesterase activity were noted at the two higher doses in males and females about 6h after dosing; females were said to have been more severely affected. At 1.6mg/kgbw in males, excessive salivation, irregular gait and tremors were observed in one male and irregular gait was observed in two males at 1.2mg/kgbw group. Seven females at 1.2mg/kgbw group exhibited irregular gait and tremors. Other findings in this group were excessive salivation and lacrimation, moist rales and ventral surface and anogenital yellow staining in two animals, and decreased activity in one animal. At a dose of 0.9mg/kgbw, the only clinical finding observed was ano-genital staining in one female. No clinical signs were

observed in other groups. Miosis was not reported for any animals in the study. Plasma cholinesterase activity was statistically significantly inhibited in males by 45%, 90% and 88% at doses of 0.40, 1.2, and 1.6 mg/kg bw per day, respectively, and in females by 17%, 87% and 96% at 0.3, 0.9, and 1.2 mg/kg bw, respectively. Erythrocyte cholinesterase activity was inhibited at the two higher doses in both sexes: in males, by 92–94% (statistically significant); and in females, by 88–93% (not statistically significant but considered to be treatment-related). Brain cholinesterase activity was statistically significantly inhibited by 57% and 52% at 1.2 and 1.6 mg/kg bw, respectively, in males. A statistically significant decrease in brain cholinesterase activity of 67% was observed in females at 1.2 mg/kg bw, and a decrease of 41% at 0.9 mg/kg bw was considered to be treatment-related, if not statistically significant. In the pilot study, no treatment-related effects were reported in either sex at doses of <0.40 mg/kg bw in males and <0.30 mg/kg bw in females.

Before the start of the definitive study, survival was assessed in an additional group of 10 females given the test material at a dose of 0.90 mg/kg bw by gavage. The only data reported for this study were the findings for animals manifesting clinical signs of toxicity during daily observations. Signs of toxicity (first observed on the day of treatment and apparently disappearing by day 5 after dosing) were reported for six of the 10 animals and included bilateral tremors in fore and hind paws, slight to moderate anogenital staining, decreased faecal volume, fasciculation, extreme lacrimation and excessive salivation. No miosis was observed.

In the definitive study, all animals were assessed for physical condition, mortality, clinical signs of toxicity, body-weight changes and food consumption. Neurobehavioural evaluations (motor activity and functional observational battery) were conducted on 10 animals of each sex per dose pre-test, at approximately 6 h after dosing (time of peak effect) and on days 8 and 15 (study termination day). Blood was collected for plasma and erythrocyte cholinesterase activity measurements from 10 animals of each sex per dose at about 6 h after dosing, and on day 8, and in five animals of each sex per dose on day 15. Brain cholinesterase activity in one-half brain homogenates was also assessed in the same number of animals at about 6 h after dosing and on day 15. Cholinesterase inhibition was determined relative to the appropriate concurrent control group value. All animals received macroscopic examinations and selected tissues from the central and peripheral nervous system were evaluated histopathologically in five perfused animals of each sex per group.

In the definitive study, the only death attributed to treatment occurred in females at the highest dose, 5–6 h after dosing. There were no accompanying clinical findings reported for this animal, which was replaced by another female for the remainder of the study. Clinical signs of toxicity, some commencing on the day of treatment, were noted in the daily physical examinations in one surviving female at the highest dose; these included moderate to extreme ano-genital staining, lethargy, decreased faecal volume and food consumption, and oral/buccal staining. No clinical findings were reported after day 5 during daily physical observations in this female. One male at the intermediate dose exhibited a red ocular exudate on day 9, but this may have been related to orbital sinus bleeding for cholinesterase measurements. Slight statistically significant decreases in body weights observed in females at the highest dose may have been largely related to decreases noted in the one animal exhibiting clinical signs of toxicity. There were no significant changes noted in male body weights or food consumption in either sex, relative to values for the respective control groups.

There were no statistically significant differences noted in the mean value for motor activity for the group, relative to values for the respective control group, on days 1, 8 and 15. The study report mentioned that the one female at the highest dose that had clinical signs of toxicity during the daily physical examinations also exhibited decreased motor activity on days 1 and 8, but not on day 15.

Treatment-related abnormalities in the functional observational battery, attributed to cholinergic toxicity, were apparent in males and females at the intermediate and highest dose and only during the peak time assessment at day 1. Miosis, present in three males at the intermediate dose, six males at the highest dose and one female at the intermediate dose, was the only finding at these doses. A wider spectrum of findings was present in females at the highest dose. In addition to miosis in all 10 rats, females in this group exhibited tremors (seven animals), and muscle fasciculations (four animals) and, to a lesser extent, ataxic gait and slightly impaired locomotion, tip-toe gait, moderate lacrimation and soiled coat. In one animal, very low arousal state, flattened posture (in home cage), no approach response, severe lacrimation and slight salivation, no open field movement, soiled coat, and markedly decreased forelimb and hindlimb grip strength were reported. At the next two time-points (days 8 and 15), no abnormal findings were evident in any group.

On day 1, around the time of peak effect, plasma cholinesterase activity was statistically significantly decreased in males by 31% and 69%, and in females by 20% and 90% at the intermediate and highest doses, respectively. There was no statistically significant inhibition at any dose in either sex on days 8 or 15. Erythrocyte cholinesterase activity was statistically significantly inhibited on day 1 only in males (67%) and females (90%) at the highest dose. Values continued to be decreased in females at the highest dose by about 45% on days 8 and 15. Brain cholinesterase activity was statistically significantly inhibited on day 1 only at the highest dose by 21% in males, and by 51% in females. Statistically significant depression of brain cholinesterase activity (of 13%) continued to be observed on day 15 in females at the highest dose.

Macroscopic and microscopic examinations did not reveal any findings related to treatment. Trauma related to retro-orbital sinus bleeding was considered to account for the minimal to slight focal degeneration of optic nerve fibres accompanied by minimal to slight gliosis observed in two females at the highest dose and minimal degeneration of a single sciatic nerve fibre in only one male at the highest dose was considered to be incidental in nature. It should be noted that miosis was observed during the functional observational battery in both sexes at 0.3 mg/kg bw, with no significant decrease in concurrently measured brain and erythrocyte cholinesterase activity. Only marginal (but statistically significant) concurrently measured plasma cholinesterase activity was observed at this dose in both sexes. If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL was 0.15 mg/kg bw in males and females on the basis of miosis in the functional observational battery in both sexes at the next highest dose (Mandella, 1998). Statements of compliance with QA and GLP were provided and the study protocol was consistent with EPA Subdivision F Guidelines (1984, revised and March 1991).

(c) *Neurotoxicity after repeated doses*

In a study of neurotoxicity, groups of 20 male and 20 female Sprague-Dawley derived (outbred) Crl:CD®(SD)IGS BR VAF/Plus® rats were given diets containing technical-grade terbufos (AC 92,100; purity, 89.7%; dissolved in acetone and mixed with a GRIT-

O'Cobs® carrier) at a dose (adjusted for purity) of 0 (diet mixed with acetone and carrier), 0.5, 0.8, or 5.0 mg/kg in males (equal to 0, 0.036, 0.059 and 0.369 mg/kg bw per day) and of 0 (diet mixed with acetone and carrier), 0.5, 0.8 or 3.0 mg/kg in females (equal to 0, 0.042, 0.064, and 0.251 mg/kg bw per day) daily for approximately 13 weeks. Animals evaluated for potential neurobehavioural changes were treated for at least 13 weeks and animals in which cholinesterase activities were measured were treated for at least 85 days. Selection of doses was based on a 22-day preliminary study of feeding in the same strain of rat (Mandella, 1999), in which treatment-related findings were noted at doses of ≥ 5 mg/kg (0.55 mg/kg bw per day) in males and ≥ 3 mg/kg (0.33 mg/kg bw per day) in females. In the 13-week study, all animals were assessed for physical condition, mortality, clinical signs of toxicity, body-weight changes, and food consumption and received ophthalmoscopic examinations. Neurobehavioural evaluations (motor activity and functional observational battery) were conducted on 10 animals of each sex per group, before treatment and at weeks 4, 8, and 13. In animals designated for cholinesterase activity determinations (9–10 animals of each sex per group), blood samples for assessment of plasma, erythrocyte and brain (one-half homogenate) cholinesterase activities were obtained at weeks 4, 8, and 13. In animals designated for neurobehavioural examinations, blood and brain samples for measurement of cholinesterase activities were obtained from five animals of each sex per group during weeks 13 or 14. Cholinesterase inhibition was determined relative to values for the appropriate concurrent control group. At study termination (week 13 or 14), macroscopic examinations were performed on all animals and selected tissues from the central and peripheral nervous system were evaluated histopathologically in five animals of each sex per group.

One male at the lowest dose died in week 5 due to accidental causes. Otherwise, there were no deaths in the study. There were no findings during physical examinations that were considered to be treatment-related and miosis was not observed in any animal. The results of ophthalmoscopic examinations were not remarkable. Over the 13-week period, males at the highest dose gained only about 90% of the weight gained by animals in the control group; a possible effect of treatment could not be dismissed. Weight gains in males at the lowest and intermediate doses during the same period were slightly higher than those of animals in the control group. Females at the highest dose gained about 13% more weight over the duration of the study than did animals in the control group, but this was not considered to be an adverse effect. There were no changes in food consumption in either sex that could definitively be ascribed to treatment and no obvious effect of treatment with the test material on motor activity or on functional observational battery parameters. In the macroscopic and microscopic examinations, there were no findings that were attributable to treatment.

At the highest dose, plasma cholinesterase activity in animals designated for cholinesterase measurements or neurobehavioural evaluations was statistically significantly inhibited at all time-points by 70–74% in males and by 91–92% in females. In the same groups, erythrocyte cholinesterase activity was virtually completely inhibited at all time-points (decreases of 97–100% in males and 100% in females). At the intermediate dose, erythrocyte cholinesterase activity was statistically significantly inhibited in males in the group of animals designated for cholinesterase measurement at week 8 and 13 by 48% and 37%, respectively, and by 35% at week 13 in the group designated for neurobehavioural evaluations. In both groups of females at the intermediate dose, inhibition of 33% (statistically significant) and 27% (not statistically significant) was observed at week 13. At study termination, brain cholinesterase activity was decreased only at the highest dose in animals

Table 4. Acute toxicity of metabolites and degradation products of terbufos in female mice

Metabolite	Strain	Route	Vehicle	LD ₅₀ (mg/kg bw)	Purity (%)	Reference
Terbufoxon sulfoxide ^a	CF1 albino	Oral	Corn oil	1.1	Not stated	American Cyanamid Company A72-35 (1972d)
Terbufos sulfoxide ^b	CF1 albino	Oral	Corn oil	3.4	Not stated	American Cyanamid Company A72-37 (1972b)
Terbufoxon sulfone ^c	CF1 albino	Oral	Corn oil	3.4	Not stated	American Cyanamid Company A72-38 (1972e)
Terbufos sulfone ^d	CF1 albino	Oral	Corn oil	14 ⁱ	Not stated	American Cyanamid Company A72-34 (1972c)
Terbufoxon ^e	CF1 albino	Oral	Corn oil	2.2	Not stated	American Cyanamid Company A72-36 (1972f)
CL 202,135 ^f	CF1 albino	Oral	Corn oil	3670 ^g	Not stated	American Cyanamid Company A73-21 (1973a)
CL 202,474 ^h	CF1 albino	Oral	Corn oil	>2500 ^j	Not stated	American Cyanamid Company A73-122 (1973b)

Although reports for these studies were not detailed and statements of compliance with GLP or QA were not provided, protocols appeared to be generally consistent with EPA Subdivision F Guidelines (1982 or 1984, revised)

^aPhosphorothioic acid, *S*-(*t*-butylsulfinyl) methyl *O,O*-diethyl ester (CL 94,365)

^bPhosphorodithioic acid, *S*-(*t*-butylsulfinyl) methyl *O,O*-diethyl ester (CL 94,301)

^cPhosphorothioic acid, *S*-(*t*-butylsulfonyl) methyl *O,O*-diethyl ester (CL 94,302)

^dPhosphorodithioic acid, *S*-(*t*-butylsulfonyl) methyl *O,O*-diethyl ester (CL 94,320)

^ePhosphorothioic acid, *S*-(*t*-butylthio) methyl *O,O*-diethyl ester (CL 94,221)

^fMethane, bis(*t*-butylsulfonyl) (CL 202,135)

^gReport stated that acute oral LD₅₀ was calculated assuming a mortality of 9 out of 10 animals at a dose of 10000 mg/kg of feed

^hMethane, (*t*-butylsulfinyl)(methylsulfinyl)

ⁱReport stated that acute oral LD₅₀ ws calculated assuming a mortality of five out of five animals at a dose of 50 mg/kg of feed

^jReport stated that animals were not fasted

designated for cholinesterase measurement and neurobehavioural evaluation, by 58% and 55%, respectively, in males and by 71% and 68%, respectively in females.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect and statistically significant inhibition of erythrocyte cholinesterase activity of 33–43% is not considered to be a relevant effect for terbufos, the NOAEL was 0.8 mg/kg (equal to 0.059 mg/kg bw per day in males and 0.064 mg/kg bw per day in females) on the basis of statistically significant inhibition of brain and erythrocyte (almost completely inhibited) cholinesterase activity at the next highest dose (Mandella, 1999). Statements of compliance with QA and GLP were provided. The protocol was consistent with EPA Subdivision F Guidelines (November, 1984, revised, and March 1991).

(d) Studies on metabolites

In a study of short-tem toxicity, groups of four male beagle dogs were given gelatin capsules containing technical-grade terbufos (AC 92,100; purity, 89.6%) or one of its metabolites, terbufos sulfoxide (CL 94,301; purity, 90.0%) and terbufos sulfone (CL 94,320; purity, 92.0%), in corn oil, administered orally once daily in the morning for at least 14 days. A similarly treated control group of six animals received gelatin capsules containing corn oil only. The animals used were described as having physical impairments but not of sufficient magnitude to jeopardize study integrity. Doses administered (adjusted for purity) were: terbufos, 2.5 or 250 µg/kg bw per day; terbufos sulfoxide, 5, 15, 625, or 250 µg/kg bw per day; or terbufos sulfone, 15, 62.5, 250, and 1000 µg/kg bw per day. A limited number of parameters were assessed, including mortality, morbidity and clinical signs of toxicity, measurement of body weight and food consumption, and a gross examination at the end of the study. Plasma and erythrocyte cholinesterase activity in fasted

animals was assessed twice before treatment, and on days 4, 8, 11, and 15. Brain cholinesterase activity (in homogenates of cerebellum and cerebrum) was measured from tissues taken at study termination (day 15 or 16) and stored frozen until analysis. Inhibition of cholinesterase activity was determined relative to the value for the appropriate concurrent control group. Overall, the data on brain cholinesterase activity, especially those from the cerebellum, were considered to be unreliable owing to inconsistencies, variability in the data and poor dose–response relationships. Clinical chemistry, haematological, urine analysis, organ weight and histopathological evaluations were not conducted in this study.

There were no deaths in the control group. The only clinical observations reported were occurrence of soft faeces (four animals) and one occurrence of alopecia. There were no other adverse findings.

There were no deaths in the groups of animals treated with terbufos. Clinical findings attributed to treatment at the highest dose (250 µg/kg bw per day) were instances of emesis and lacrimation, and an increased incidence of soft or soft sanguineous-looking faeces (seven occurrences) compared with the control group; treatment-related decreases in body weight and food consumption were also noted in some animals. At the highest dose, plasma cholinesterase activity was statistically significantly inhibited by 57–76% at all time-points during the study, and erythrocyte cholinesterase activity was statistically significantly inhibited by 42–79% on days 8, 11 and 15, and cerebellar and cerebral cholinesterase activities were statistically significantly inhibited by 48% and 37%, respectively. At the lowest dose, statistically significant decreases in plasma cholinesterase activity were noted on days 11 and 15, but they were of low magnitude (23–25%) and not clearly related to treatment. There were no obvious effects of treatment on erythrocyte or cerebral cholinesterase activities at the lowest dose. Cerebellar cholinesterase activity at the lowest dose was statistically significantly inhibited by 42%. As a finding of significant brain cholinesterase inhibition at this dose was inconsistent with other studies of repeated dosing with terbufos, it was considered highly unlikely to be related to treatment. A finding of dark mesenteric lymph node(s) at gross necropsy in one animal in the group treated with the lowest dose of terbufos was not clearly related to treatment.

In the groups of animals treated with terbufos sulfoxide, there were no mortalities. At the highest dose (250 µg/kg bw per day), clinical findings ascribed to treatment included ataxia, salivation, languid behaviour, salivation, miosis, no faeces, emesis, and an increased incidence relative to control of soft faeces (11 occurrences). Decreases in body weight and food consumption attributed to treatment were noted in some animals at the highest dose.

At all time-points during the study, plasma and erythrocyte cholinesterase activities were statistically significantly inhibited by 60–71% and 30–93%, respectively, at the highest dose. In groups given terbufos sulfoxide at 62.5 and 15 µg/kg bw per day, there was no clear treatment-related effect on body weights, food consumption or clinical findings. Single occurrences of emesis or sanguineous-looking emesis were noted in each of these groups, as was one instance of no faeces at 15 µg/kg bw per day. Occurrences of soft stool in the control group, and at the lowest, lower intermediate, higher intermediate and highest dose were 4, 3, 13, 6, and 11, respectively. Increases relative to the control group at the two intermediate doses did not appear to be part of an obvious pattern that might be definitively attributed to treatment. Although plasma cholinesterase activity was statistically significantly inhibited at all time-points at both intermediate doses, erythrocyte enzyme activity was not statistically significantly inhibited at any time-point at either dose. Inhibition of brain

cholinesterase activity was found at the two higher doses. Cerebral cholinesterase activity was statistically significantly inhibited only at the highest dose (250 µg/kg bw per day) by 45%. At the higher intermediate and highest doses, cerebellar cholinesterase activity was statistically significantly inhibited by 52% and 31% (not statistically significant), respectively. At the lowest dose (5.0 mg/kg bw per day), there were no obvious effects of treatment on the parameters assessed. A statistically significant decrease in plasma cholinesterase activity that was noted only on day 15 was of low magnitude (19%). Findings at gross necropsy that were reported to be related to treatment were observed in one dog at the highest dose. They were described as a dark red area in the mucosa of the jejunum mucosa, associated with dark red mesenteric lymph node(s). This animal exhibited a number of clinical signs during the study and had decreases in plasma, erythrocyte cerebellar and cerebral cholinesterase activities. Dark mesenteric lymph node(s) were also reported in one other animal at the highest dose.

In groups of animals treated with terbufos sulfone, there were three treatment-related deaths preceded by clinical signs of toxicity. Two animals were found dead on day 9 and 14 and the third was terminated in a moribund condition on day 15. Clinical findings associated with treatment in this group included tremor, languid behaviour, prostration, ataxia, emesis, salivation, dyspnoea, cold-to-touch, squint eye, miosis, no faeces, an increase in soft faeces relative to the control group, prolapsed rectum, sanguineous-appearing material in cage. Also at this dose, decreases in body weight and food consumption attributed to treatment were noted in some animals. In groups given terbufos sulfone at 250, 62.5 or 15 µg/kg bw per day, there was no clear treatment-related effect on body weights, food consumption or clinical findings. Two occurrences of lacrimation and two occurrences of soft faeces were reported at 250 µg/kg bw per day, two instances of soft or sanguineous-looking faeces and one instance of emesis were found at 62.5 µg/kg bw per day and four occurrences of soft faeces were found at the lowest dose. Plasma cholinesterase activity was statistically significantly inhibited at all time-points: by 67–77% at the highest dose, by 39–56% at 250 µg/kg bw per day, and by 24–37% at 62.5 µg/kg bw per day. At the lowest dose (15 µg/kg bw per day), statistically significant inhibition of plasma cholinesterase activity (of only 20%) was seen only on day 8 but not on day 15 and thus was not clearly related to treatment. Statistically significant decreases in erythrocyte cholinesterase activity were observed only at the highest dose where inhibition was noted at all time-points ranging from 57% to 93%. Inhibition of brain cholinesterase activity was found at the two higher doses (statistically significantly only at the highest dose, by 62%). Cerebellar cholinesterase activity was inhibited by 23% (not statistically significant) at 250 µg/kg bw per day and by 46% (not statistically significant) at the highest dose.

At gross necropsy, findings related to treatment were observed in the group receiving the highest dose: dark, red or dark red areas, intussusceptions, and/or a prolapsed anus with associated redness in the mesenteric lymph nodes were observed in the gastrointestinal tract of the animals that died. Some other gross findings at this dose were ascribed to the poor condition of the animals.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL for terbufos was 2.5 µg/kg bw per day in males on the basis of clinical findings and statistically significant inhibition of erythrocyte cholinesterase activity.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL for terbufos sulfoxide was 15.0 µg/kg bw per day in males on the basis of

statistically significant inhibition of brain cholinesterase (cerebellum) at the dose above, for which a relationship to treatment could not be dismissed.

If inhibition of plasma cholinesterase activity is not considered to be an adverse effect, the NOAEL for terbufos sulfone was 62.5 µg/kg bw per day on the basis of inhibition of brain cholinesterase (cerebellar) activity at the dose above, for which a relationship to treatment could not be dismissed (Bailey, 1988). Statements of compliance with QA and GLP were provided. This study was not conducted to fulfil a particular guideline.

3. Observations in humans

There have been a number of reports of occupational and non-occupational poisoning incidents associated with exposure to terbufos. With regard to possible effects from terbufos manufacturing facilities, no "reportable incidents" have been noted and no other information was available.

Comments

In rats, absorption of single doses of [¹⁴C]terbufos was rapid and fairly complete. Most of the radiolabel was excreted within 24–48 h. Most (about 70–80%) of the administered dose was excreted in the urine. Terbufos was extensively metabolized and little radioactivity was found in the tissues. There were no significant sex-specific differences in the toxicokinetics of terbufos.

Sulfoxidation and desulfuration of terbufos is followed by hydrolysis of the thiophosphorus bond (S–P), enzymatic *S*-methylation and then additional *S*-oxidation. On the basis of a 14-day study of repeated dosing, terbufos showed little potential for bioaccumulation.

By analogy with other phosphorodithioate compounds, it is likely that terbufos is metabolically activated to terbufos oxon and other oxons, which cause inhibition of acetylcholinesterase activity.

Terbufos is of very high acute toxicity when administered by oral, dermal, and inhalation routes. Acute oral LD₅₀ values in rodents and dogs were similar, ranging from 1.4 to 9.2 mg/kg bw. Clinical signs observed were those typical of cholinergic toxicity.

The acute dermal LD₅₀ for terbufos was about 1 mg/kg bw in rabbits; a single application of undiluted terbufos to the shaved skin (0.25–0.5 ml) or into the conjunctival sac (0.1 ml) killed all animals within 24 h. The acute LC₅₀ for terbufos administered by inhalation ranged from 0.0012 to 0.0061 mg/l in rats.

In studies in rats and dogs, the critical effects of repeated doses of terbufos were inhibition of brain cholinesterase activity and associated clinical signs. NOAELs for inhibition of brain cholinesterase activity in these studies ranged from about 0.04 to 0.11 mg/kg bw per day, and LOAELs ranged from about 0.085 to 0.55 mg/kg bw per day. Inhibition of brain cholinesterase activity of 7–12%, in the absence of clinical signs, was not considered to be toxicologically relevant. NOAELs and LOAELs for inhibition of erythrocyte cholinesterase activity were not substantially different from those for inhibition of brain cholinesterase activity.

In a 1-year study in dogs given terbufos in capsules, the NOAEL was 0.060 mg/kg bw per day on the basis of inhibition of brain acetylcholinesterase activity at 0.090 mg/kg bw per day. The NOAELs for inhibition of brain acetylcholinesterase activity in other short-term studies in dogs were consistent with that of the 1-year study.

In an 18-month study in mice fed with terbufos, there was no evidence of carcinogenicity at doses considered relevant for risk assessment. Cholinesterase activities were not measured. The NOAEL was 3 mg/kg (equivalent to 0.45 mg/kg bw per day) on the basis of significant decreases in body weights at the next highest dose of 6 mg/kg (equivalent to 0.9 mg/kg bw per day).

A 2-year study of toxicity and carcinogenicity in rats had limitations that included outstanding questions involving the etiology and/or relationship to treatment of certain non-neoplastic findings, confounding by non-treatment related illness in test animals and the lack of supporting data to adequately quantify dietary intake, stability, and homogeneity. However, on the basis of inhibition of brain cholinesterase activity in animals receiving the highest dose, this study was considered to be adequate for testing for carcinogenicity. No increase in tumour incidence was observed after a histopathological re-evaluation of tissues for the assessment of carcinogenic potential. The NOAEL was 1 mg/kg (equivalent to 0.05 mg/kg bw per day) on the basis of inhibition of brain acetylcholinesterase activity at 4 mg/kg (equivalent to 0.2 mg/kg bw per day).

This conclusion was supported by a subsequent 1-year study of toxicity in rats; no significant systemic or neoplastic effects were observed. The NOAEL was 1 mg/kg (equal to 0.055 mg/kg bw per day) on the basis of the absence of significant inhibition of brain acetylcholinesterase activity at all doses.

The Meeting concluded that terbufos was not carcinogenic in mice or rats.

The genotoxic potential of terbufos was assessed in an adequate range of in vitro and in vivo tests. On the basis of the overall weight of evidence from the studies of genotoxicity, the Meeting concluded that terbufos is unlikely to pose a genotoxic risk to humans.

In view of the lack of significant genotoxicity and the absence of carcinogenicity observed, the Meeting concluded that terbufos is unlikely to pose a carcinogenic risk to humans.

In a study of reproductive toxicity in rats, mortality and clinical signs of toxicity in females, some occurrences of excess salivation in males and decreases in body weight and food consumption in both sexes were observed at 5 mg/kg (equal to 0.42 mg/kg bw per day), a treatment that was terminated prematurely. At 2.5 mg/kg, an increase in soft stools and body-weight loss was noted in lactating females. Also observed were decreases in pregnancy rate, male fertility and significant decreases in the mean body weight of viable pups on days 14 and 21 of lactation in F₀ and F₁ litters. The NOAEL for effects on reproduction and offspring was 1.0 mg/kg (equal to 0.086 mg/kg bw per day). Inhibition of brain cholinesterase activity was observed in both sexes, with a NOAEL of 0.5 mg/kg (equal to 0.043 mg/kg bw per day).

In a study of developmental toxicity in rats, the NOAEL for maternal and developmental effects was 0.2 mg/kg bw per day, the highest dose tested. Mortality was seen at a dose of 0.4 mg/kg bw per day in a preliminary study.

In a study of developmental toxicity in rabbits, the NOAEL for maternal and developmental effects was 0.25 mg/kg bw per day on the basis of clinical and systemic findings in does, an increased number of does with resorption sites and decreased fetal body weights at the next highest dose of 0.50 mg/kg bw per day.

The potential of terbufos to induce delayed polyneuropathy in hens when given as a single dose by gavage was assessed. The activity of neuropathy target esterase was not measured in this study. No significant changes in spinal cord and peripheral nerves were apparent in the group treated with terbufos. The Meeting concluded that at doses relevant to dietary exposure in humans, there was no concern for the induction of delayed polyneuropathy by terbufos.

In a study of neurotoxicity in which terbufos was given to rats as a single dose by gavage, mortality, clinical signs of toxicity, including miosis, and inhibition of brain and erythrocyte cholinesterase activities were noted at the highest dose tested of 0.90 mg/kg bw. The only finding at the intermediate dose (0.3 mg/kg bw) was miosis, which was observed in the absence of inhibition of concurrently measured brain and erythrocyte cholinesterase activities. No neurohistopathological lesions were found at any dose. The NOAEL was 0.15 mg/kg bw on the basis of findings of miosis in both sexes at the next highest dose of 0.30 mg/kg bw.

A 13-week study of neurotoxicity was conducted in rats. Other than a slight decrease in body weight and inhibition of brain and erythrocyte cholinesterase activities at the highest dose of 3.0 mg/kg (equal to 0.25 mg/kg bw per day), no effects (including miosis) were observed. The NOAEL was 0.8 mg/kg (equal to 0.059 mg/kg bw per day) on the basis of inhibition of brain acetylcholinesterase activity at 0.25 mg/kg bw per day.

The acute oral toxicity of a number of metabolites of terbufos was evaluated in female mice. LD₅₀s were as follows: 1.1 mg/kg bw (terbufoxon sulfoxide), 3.4 mg/kg bw (terbufos sulfoxide), 3.4 mg/kg bw (terbufoxon sulfone), 14 mg/kg bw (terbufos sulfone), 2.2 mg/kg bw (terbufoxon), 3670 mg/kg bw (methane, bis (*tert*-butylsulfonyl) and >2500 mg/kg bw (methane, (*tert*-butylsulfinyl)(methylsulfinyl)).

In a comparative 14-day study in dogs, terbufos given in capsules was found to be more toxic than either terbufos sulfoxide or terbufos sulfone.

There have been a number of reports of occupational and non-occupational poisoning incidents associated with exposure to terbufos. No information was available regarding possible effects from terbufos manufacturing facilities.

The Meeting concluded that the existing database on terbufos was adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.0006 mg/kg bw based on an overall NOAEL of 0.06 mg/kg bw per day and a safety factor of 100 for inhibition of brain cholinesterase activity, in the 1-year study of toxicity, the 13-week study of neurotoxicity and the two-generation study of reproduction in rats, and the 1-year study in dogs.

The Meeting established an acute reference dose (RfD) of 0.002 mg/kg bw based on a NOAEL of 0.15 mg/kg bw per day for miosis in the study of neurotoxicity in rats given a single dose of terbufos, and a 100-fold safety factor. Since only in this study was miosis observed in the absence of inhibition of cholinesterase activity, it might be possible to refine the acute RfD after better characterization of this effect.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of toxicity and carcinogenicity ^a	Toxicity	3 mg/kg of feed, equivalent to 0.45 mg/kg bw per day	6 mg/kg of feed, equivalent to 0.90 mg/kg bw per day
		Carcinogenicity	12 mg/kg of feed, equivalent to 1.8 mg/kg bw per day ^d	—
Rat	2-year study of toxicity and carcinogenicity ^a	Carcinogenicity	4 mg/kg of feed, nominally equivalent to 0.2 mg/kg bw per day ^d	—
	1-year study of toxicity ^a	Toxicity	1 mg/kg of feed, equal to 0.055 mg/kg bw per day ^d	—
	13-week study of neurotoxicity ^a	Toxicity	0.8 mg/kg of feed, equal to 0.059 mg/kg bw per day	3.0 mg/kg of feed, equal to 0.25 mg/kg bw per day
	Single-dose study of neurotoxicity ^b	Toxicity	0.15 mg/kg bw per day	0.30 mg/kg bw per day
	Multigeneration study of reproductive toxicity ^a	Parental toxicity	0.5 mg/kg of feed, equal to 0.043 mg/kg bw per day	1.0 mg/kg of feed, equal to 0.086 mg/kg bw per day
		Offspring toxicity	1.0 mg/kg of feed, equal to 0.086 mg/kg bw per day	2.5 mg/kg of feed, equal to 0.21 mg/kg bw per day
	Study of developmental toxicity ^b	Maternal toxicity	0.20 mg/kg bw per day ^d	—
		Embryo- and fetotoxicity	0.20 mg/kg bw per day ^d	—
Rabbit	Study of developmental	Maternal toxicity	0.25 mg/kg bw per day	0.50 mg/kg bw per day
Dog	1-year study of toxicity ^c	Embryo- and fetotoxicity	0.25 mg/kg bw per day	0.50 mg/kg bw per day
		Toxicity	0.06 mg/kg bw per day	0.09 mg/kg bw per day

^a Diet
^b Gavage
^c Capsule
^d Highest dose tested

Estimate of acceptable daily intake for humans

0–0.0006 mg/kg bw

Estimate of acute reference dose

0.002 mg/kg bw

Studies that would provide information useful for continued evaluation of the compound

- A study of delayed neurotoxicity with neuropathy target esterase measurements (known to be ongoing)
- Further observations in humans
- Characterization of miosis

Summary of critical end-points for terbufos

<i>Absorption, distribution, excretion and metabolism in mammals</i>			
Rate and extent of oral absorption		Rapid and fairly complete	
Dermal absorption		No specific study; rapidly penetrating following dermal or ocular application	
Distribution		Relatively rapid and fairly complete	
Potential for accumulation		Little	
Rate and extent of excretion		Relatively rapid and complete; most eliminated in 24–48 h; elimination in urine predominates	
Metabolism in animals		Sulfoxidation and desulfuration of terbufos is followed by hydrolysis of the thiophosphorus bond (S-P), enzymatic S-methylation and then additional S-oxidation	
Toxicologically significant compounds		Terbufos Terbufos oxon Terbufos sulfoxide Terbufos sulfone Terbuoxon sulfoxide Terbuoxon sulfone	
<i>Acute toxicity</i>			
Rat, LD ₅₀ , oral		1.4–9.0 mg/kg bw	
Rabbit, LD ₅₀ , dermal		0.81–0.93 mg/kg bw	
Rat, LC ₅₀ , inhalation		Vapour, 4-h whole body exposure: 0.0012–0.0061 mg/l	
Dermal irritation		Could not be determined due to lethality, rabbit	
Ocular irritation		Could not be determined due to lethality, rabbit	
Skin sensitization		Not determined owing to potential for severe toxicity	
<i>Short-term studies of toxicity</i>			
Target/critical effect		Inhibition of brain cholinesterase activity	
Lowest relevant oral NOAEL		0.059 mg/kg bw per day (13-week study of neurotoxicity in rats)	
Lowest relevant dermal NOAEL		Data not available	
Lowest relevant inhalation NOAEC		No appropriate data available	
Genotoxicity		Unlikely to be genotoxic	
<i>Long-term studies of toxicity and carcinogenicity</i>			
Target/critical effect		Inhibition of brain cholinesterase activity	
Lowest relevant NOAEL		0.055 mg/kg bw per day (1-year study in rats)	
Carcinogenicity		No evidence of carcinogenicity; Unlikely to pose a risk to humans	
<i>Reproductive toxicity</i>			
Reproduction target/critical effect		Decreases in male fertility and female pregnancy rate	
Lowest relevant reproductive NOAEL		0.086 mg/kg bw per day (rats)	
Developmental target/critical effect		Not teratogenic; Reduced fetal body weight	
Lowest relevant developmental NOAEL		0.25 mg/kg bw per day (rabbits)	
<i>Neurotoxicity/delayed neurotoxicity</i>			
Acute neurotoxicity			
Target/critical effect		Miosis	
Relevant NOAEL		0.15 mg/kg bw (rats)	
13-week study of neurotoxicity			
Target/critical effect		Inhibition of brain cholinesterase activity	
Relevant NOAEL		0.059 mg/kg bw per day (rats)	
Delayed neuropathy		No evidence to suggest toxicity at dietary exposures	
Medical data		There have been a number of reports of occupational and non-occupational poisoning incidents associated with exposure to terbufos. No information was available regarding possible effects from terbufos manufacturing facilities.	

Summary	Value	Study	Safety factor
ADI	0–0.0006 mg/kg bw	Rats and dogs, overall NOAEL for studies of repeated doses	100
Acute RfD	0.002 mg/kg bw	Rat, study of acute neurotoxicity	100

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ANNEX 1

Reports and other documents resulting from previous Joint Meetings Of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and WHO Expert Groups on Pesticide Residues

1. Principles governing consumer safety in relation to pesticide residues. Report of a meeting of a WHO Expert Committee on Pesticide Residues held jointly with the FAO Panel of Experts on the Use of Pesticides in Agriculture. FAO Plant Production and Protection Division Report, No. PL/1961/11; WHO Technical Report Series, No. 240, 1962.
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3. Evaluation of the toxicity of pesticide residues in food. Report of the Second Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1965/10; WHO/Food Add./26.65, 1965.
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This volume contains toxicological monographs that were prepared by the 2003 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Geneva from 15 to 24 September, 2003.

The monographs in this volume summarize the safety data on 13 pesticides that could leave residues in food commodities. These pesticides are carbosulfan, cyprodinil, dimethoate, famoxadone, malathion, methoxyfenozide, paraquat, phosmet, pyraclostrobin, pyrethrins, tebufenozide and terbufos. The data summarized in the toxicological monographs served as the basis for the acceptable dietary intakes and acute reference doses that were established by the Meeting.

This volume and previous volumes of JMPR toxicological evaluations, many of which were published in the FAO Plant Production and Protection Paper series, contain information that is useful to companies that produce pesticides, government regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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